The Role of Tyrosine Rich Acidic Matrix Protein in the Extracellular Matrix.

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Abstract

Tyrosine rich acidic matrix protein (TRAMP) is a 22 kilodalton protein that co-purifies with lysyl oxidase from porcine skin. Human and bovine equivalents have also been identified. The biological significance of TRAMP in the extracellular matrix is not understood, although the bovine form has been shown to interact with the dermatan sulphate proteoglycan, decorin and alter its effects on cell adhesion. Previously, TRAMP has been shown to accelerate collagen I fibril formation *in vitro* at sub-stoichiometric molar ratios of TRAMP to collagen.

A number of purification strategies were performed in an attempt to produce highly purified, active TRAMP free from lysyl oxidase for fibrillogenesis and enzymatic studies. Unsuccessful methods included hydrophobic interaction chromatography, affinity purification on amino-hexyl Sepharose and preparative isoelectric focusing. TRAMP was eventually purified by DEAE anion exchange followed by Superdex-75 size exclusion (in the presence of urea) and Mono Q FPLC anion exchange chromatography. Yields of 10μg of TRAMP per g wet weight starting material were obtained.

TRAMP purified as above was active in fibrillogenesis assays using the 'warm start' technique, in buffers containing 30mM TES, 30mM Na_2HPO_4, 135mM NaCl pH 7.4. The acceleratory effect of TRAMP on collagen I fibril formation was also observed when the phosphate concentration was lowered to 10mM and when TES was removed. TRAMP has previously been shown to bind to *in vitro* reconstituted collagen fibrils if present during their formation. Reducing the phosphate concentration decreased the amount of TRAMP associated with collagen I fibrils in co-sedimentation assays, whilst subsequent removal of TES had no effect on TRAMP binding to collagen I fibrils. Preliminary observations also suggested that treatment of TRAMP with sulphatase had no effect on the ability of TRAMP to accelerate collagen I fibril formation. A solid phase assay showed TRAMP to bind collagen I monomers with a higher affinity than fibrils suggesting a role for TRAMP in the early, nucleation phase of fibril formation. TRAMP was unable to reverse the inhibitory effect of decorin on fibril formation. The presence of decorin had no effect on

TRAMP binding to collagen monomers in solid phase assays but led to an increase in the amount of TRAMP associated with collagen fibrils in co-sedimentation assays. Attempts to identify specific binding sites for TRAMP on *in vitro* reconstituted collagen I fibrils by immunogold labelling and electron microscopy were unsuccessful.

Western blot analysis of murine tissues confirmed previous reports that TRAMP was present in lung and skeletal muscle and absent from brain and spleen. In addition, TRAMP was found in bladder, ovary, testes and duodenum. Immunocytochemistry of 21 day mouse tissues with affinity purified antibody to porcine TRAMP showed it to be present in the dermis of the skin, surrounding skeletal muscle fibres, within the myocardium, endocardium and supporting tissue of the heart, in the sclera of the eye and within the intestinal microvilli in duodenum. Previous amino acid sequence analysis suggested that TRAMP may be a copper-dependent amine oxidase, due to the presence of the putative topaquinone consensus sequence N-Y-D. However, TRAMP showed no amine oxidase activity on either benzylamine or [4,5-^{3}H] lysine labelled elastin or collagen substrates.

It is proposed that TRAMP plays an important role in the early, nucleation stages of fibril formation by binding two adjacent collagen monomers and thus stabilising the nuclei. The presence of TRAMP at the end of the growing fibril may also serve to direct cells to sites for collagen deposition.

Declaration

I declare that all material presented in this thesis, unless stated otherwise, is the sole work of the author, as is the composition.

Harriet M. Bear

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Abbreviations

ACP	Aldol condensation product
β-APN	β-aminopropionitrile
BCA	Bicinchonicic acid
BMP-1	Bone morphogenetic protein-1
BSA	Bovine Serum Albumin
BSAO	Bovine Serum Amine Oxidase
Bis-Tris	(Bis[2-hydroxyethyl]imino-tris[hydroxymethyl]methane)
CAO	Copper amine oxidase
cDNA	complementary deoxyribonucleic acid
CO_2	Carbon dioxide
cpm	Counts per minute
CS	Chondroitin sulphate
DEAE	Diethylaminoethyl
dH_2O	Distilled water
dpm	Disintegrations per minute
DS	Dermatan sulphate
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
FACIT	Fibril associated collagen with interrupted triple helices
FPLC	Fast protein liquid chromatography
GAG	Glycosaminoglycan
^3H	Tritium
HCl	Hydrochloric acid
HIC	Hydrophobic interaction chromatography
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
HS	Heparan sulphate
IEF	Isoelectric focusing
KS	Keratan sulphate
LRR	Leucine-rich repeat
LTQ	Lysine tyrosylquinone
MBq	Megabecquerels
MEM	Minimum essential medium
$MgCl_2$	Magnesium chloride
μCi	Microcurie
mRNA	messenger ribonucleic acid
NEM	N-ethylmaleimide
PCPE	Procollagen C-proteinase enhancer
PB	Phosphate buffer
PBS	Phosphate buffered saline
PBU	Phosphate buffered urea
pI	Isoelectric point
PMSF	Phenylmethylsulphonyl fluoride
PPQ	Pyroloquinoline
SDS	Sodium dodecyl sulphate

SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SLRP	Small leucine-rich proteoglycan
TBS	Tris buffered saline
TBST	Tris buffered saline-Tween
TBU	Tris buffered urea
TCA	Trichloroacetic acid
TEMED	N',N',N',N', tetramethylethylenediamine
TES	N-[tris(hydroxymethyl)methyl-2-amino]-ethanesulphonic acid
TRAMP	Tyrosine rich acidic matrix protein
Tris	Tris(hydroxymethyl)aminomethane
TPQ	Topaquinone
Tween-20	Polyoxyethylenesorbitan monolaurate
V_e	Elution volume
V_o	Void volume
V_t	Total volume

Amino Acids

Single letter code	Three letter code	Amino acid
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

Chapter 1

Introduction

1.1 The Extracellular Matrix

The extracellular matrix (ECM) is an organised meshwork surrounding cells in tissues. The components of this intercellular network are synthesised by the cells to provide them with structural support and protection from external physical stress. The relationship between the cells and the matrix is dynamic. The ECM interacts with the cells to regulate their shape, growth, differentiation and migration. In return, the cells modulate the composition and organisation of the ECM.

The variations in relative amounts of the ECM components and the way in which they are organised give an amazing diversity of forms, highly adapted to the functional requirements of the particular tissue. Understanding of the complex interactions taking place to achieve such diversity is incomplete. The following review discusses some of the various components and their roles and then goes on to discuss what is known about interactions between them. For additional information on matrix glycoproteins and other ECM components see Yamada (1991) and Ayad *et al.* (1994).

1.2 Collagen

Collagen comprises a third of the total protein mass in vertebrates. It is not surprising therefore, that it is also one of the major components of the extracellular matrix, with roles in structural support, cell adhesion and cell migration (Brown and Timpl, 1995).

The general definition of a collagen is that it is a protein containing one or more domains comprised of 3 polypeptide chains folded to form a right-handed super triple helix, which is able to form supramolecular assemblies in the extracellular space. At the amino acid level each of these polypeptide chains consists of a repeating Gly-X-Y unit where X is commonly proline and Y commonly hydroxyproline. It also contains a high proportion of hydroxylysine residues. The polypeptide chains, also known as α chains, may or may not be identical (Kielty *et al.*, 1993).

1.2.1 Classification of Collagen Types

Collagen is not a single molecule but a large heterogeneous class of molecules with a tissue specific distribution (Table 1.1). Over 18 different collagen types have been identified (for reviews see Hulmes, 1992; Mayne and Brewton, 1993; Kadler, 1994; Prockop and Kivirikko, 1995). These were originally classified in order of discovery, using a numbering system with Roman numerals for collagen type and Arabic numerals for individual polypeptide or α-chains. Collagens can also be classified according to their supramolecular structure into fibrillar and non-fibrillar collagens (Hulmes, 1992). The fibrillar collagens (types I, II, III, V and XI) form rod-like fibrils with a characteristic 64-67nm (D-) repeating banding pattern when viewed by electron microscopy. The banding pattern is the result of the arrangement of neighbouring collagen molecules in a quarter-staggered array (Figure 1.1; Kadler, 1994). The most abundant and well characterised of the fibrillar collagens is collagen I which is found in skin, tendon, bone, cornea and the vascular system. Collagen II is the major cartilage collagen and is also found in embryonic cornea and vitreous humour whilst collagen III has a widespread tissue distribution similar to that of collagen I (for review see Hulmes, 1992). Collagen V forms heterotypic fibrils with collagen I and thus has a similar tissue distribution (Birk *et al.*, 1988; Birk and Lisenmayer, 1994). In a similar manner, collagen XI is localised to tissues containing collagen II (Mendler *et al.*, 1989).

The non-fibrillar collagens can be further sub-divided into the Fibril Associated Collagens with Interrupted Triple helices or FACIT (collagens IX, XII and XIV), the network forming collagens (IV and VII), a microfibrillar (beaded) collagen (VI), the short chain collagens (VIII and X) and two new sub-groups, the transmembrane collagens (XVII and XIII) and the MULTIPLEXINS (XV and XVIII; multiple triple helix domains with interruptions; Hulmes, 1992; Pihlajaniemi and Rehn, 1995). FACIT collagens have 'multiple domains' and as the name suggests are localised at specific sites on the surface of collagen fibrils (Shaw and Olsen, 1991). For example, collagen IX is covalently linked to collagen II (van der Rest and Mayne, 1988). It is postulated that FACIT collagens may form a link between fibrillar collagens and

Type	α chain(s)	Supramolecular structure	Tissue distribution: some examples
I	α1(I), α2(I)	Fibrillar	Bone, skin, cornea, lung, tendon
II	α1(II)	Fibrillar	Cartilage, vitreous humour
III	α1(III)	Fibrillar	Skin, lung, vascular system,
IV	α1(IV), α2(IV), α3(IV), α4(IV), α5(IV)	Network	Basement membrane
V	α1(V), α2(V), α3(V)	Fibrillar	Collagen I containing tissue
VI	α1(VI), α2(VI), α3(VI)	'Beaded' microfibrils	Most connective tissues
VII	α1(VII)	Anchoring fibrils	Basement membrane (skin, oral mucosa, cervix)
VIII	α1(VIII), α2(VIII)	Hexagonal arrays	Descemet's membrane, endothelial cells
IX	α1(IX), α2(IX), α3(IX)	Surface of collagen II fibrils	Collagen II containing tissues
X	α1(X)	Hexagonal arrays	Hypertrophic and mineralising zones of cartilage
XI	α1(XI), α2(XI), α3(XI)	Fibrillar, core of collagen II fibrils	Collagen II containing tissue
XII	α1(XII)	? associated with collagen I fibrils	Collagen I containing tissue
XIII	α1(XIII)	? transmembrane	most connective tissues?
XIV	α1(XIV)	? associated with collagen I fibrils	Collagen I containing tissue
XV	α1(XV) only?	unknown	Widespread
XVI	α1(XVI) only?	unknown	Human fibroblasts (from cDNA)
XVII	α1(XVII) only?	transmembrane	Human skin (from cDNA)
XVIII	α1(XVIII) only?	unknown	Widespread, most abundant in liver and lung

Table 1.1. Vertebrate collagens.

References: Types I-XIV (Hulmes, 1992; Brown and Timpl, 1995), Types XV-XVIII (Mayne and Brewton, 1993), Types XV, XVII and XVIII (Pihlajameini and Rehn, 1995).

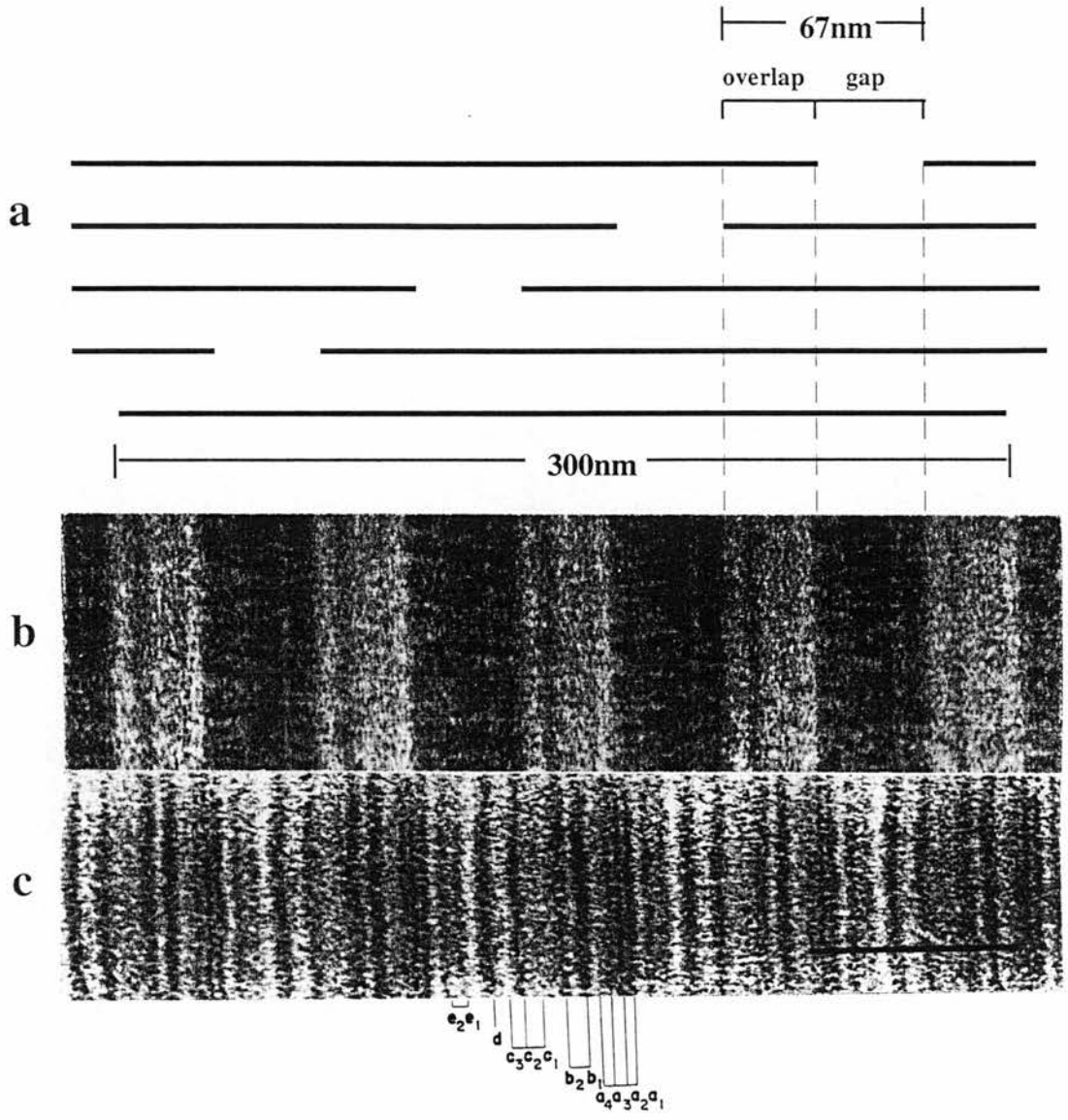


Figure 1.1. Schematic diagram illustrating the relationship between the staggered arrangement of collagen monomers within a fibril (a) and the banding patterns observed by electron microscopy of negatively (b) and positively (c) stained collagen fibrils. Electron micrographs were reproduced from Mould *et al.* (1990). For negative staining (b) fibrils were stained with 1% phosphotungstic acid, (pH 7.4) which permeates the fibrils and becomes trapped within the 'gap' zones. For positive staining (c) fibrils were stained with 1% phosphotungstic acid, (pH 2.2), followed by by 1% uranyl acetate, (pH 4.4). The banding pattern observed after positive staining is due to binding of stain to regions rich in polar amino acids (Linsenmayer, 1991).

other matrix components. The network forming collagens are found close to or as part of basement membranes, which are extracellular sheet-like structures underlying epithelial and endothelial cells (van der Rest and Garrone, 1991). Collagen VI has a widespread distribution amongst connective tissues and forms fibrils with 110nm periodicity. The microfibrillar 'beads on a string' appearance of collagen VI is due to the anti-parallel association of molecules into dimers which can then laterally associate into tetramers (Timpl and Engel, 1987). Short chain collagen molecules are approximately half the length of the fibrillar collagens and form highly organised supramolecular hexagonal arrays. The hexagonal array formed by collagen X is believed to act as a scaffold during the replacement of cartilage by bone in hypertrophic bone (Schmid and Linsenmeyer, 1990). The classification of collagens XIII and XVII as transmembrane collagens was originally based on their cDNA sequences and to date, this is the only evidence that collagen XIII is embedded in the membrane. By contrast, immunolocalisation studies have confirmed that collagen XVII is a non-secreted transmembrane protein (Hopkinson and Jones, 1996). The MULTIPLEXINS are characterised by multiple non-collagenous domains within collagenous sequences and also a unique pattern of four cysteine residues in the C-terminal non-collagenous domain. They have a widespread distribution throughout connective tissues but little is known about their structure or functions since they have yet to be characterised at the protein level (Rehn and Pihlajaniemi, 1996).

1.2.2 Post-translational modifications of collagen I

Collagen I is synthesised as individual procollagen chains and undergoes a number of post-translational modifications within the endoplasmic reticulum prior to secretion from the cell. These include hydroxylation of proline and lysine residues to hydroxyproline and hydroxylysine respectively, glycosylation of hydroxylysine residues and, finally, chain association and disulphide bonding. The collagen molecule is secreted into the extracellular matrix as a soluble precursor molecule (procollagen) containing a central triple helical domain of 300nm in length and also amino and carboxyl terminal propeptides (Kivirikko and Myllyla, 1985; Kadler, 1994). These propeptide regions are believed to be important for initiation of triple

helix formation, and maintaining the solubility of the precursor molecule (Engel and Prockop, 1991). Removal of the propeptides by specific procollagen N- and C-proteinases reduces the solubility of the collagen molecule and initiates the assembly of collagen monomers into fibrils (Section 1.7.2). Both these proteinases are neutral, zinc/calcium-dependent metalloproteinases (Hojima *et al.*, 1985; 1989). The procollagen I N-proteinase has been isolated as a 500kDa complex from chicken tendon and as a 107kDa monomer from bovine skin (Hojima *et al.*, 1989; Colige *et al.*, 1995). It only cleaves the amino propeptide from native, triple helical procollagen (Kohn *et al.*, 1974; Tuderman *et al.*, 1978). In contrast, the monomeric, 100kDa procollagen I C-proteinase is able to cleave the carboxyl propeptide from both native and denatured substrates (Goldberg *et al.*, 1975; Hojima *et al.*, 1985). The activity of the C-proteinase is increased in the presence of a 55kDa procollagen C-proteinase enhancer glycoprotein (PCPE; Adar *et al.*, 1986). Also of interest is the recent discovery that the C-proteinase is identical to bone morphogenetic protein I (BMP-I; Kessler *et al.*, 1996; Li *et al.*, 1996; Wozney *et al.*, 1988). Whether the effect of C-proteinase/BMP-1 on bone morphogenesis is due to its C-proteinase activity or an as yet undefined property of the enzyme remains to be determined. In addition, recent evidence from the cDNA sequence of procollagen I N-proteinase, suggests that it may also play a role in development (Colige *et al.*, 1997).

The action of the N- and C-proteinases does not remove all the non-helical regions from the collagen monomer. Small telopeptides remain at the N- and C-termini which are important not only for assembly into fibrils but also because they contain lysine residues on which lysyl oxidase acts to initiate the cross-linking of neighbouring molecules to stabilise collagen fibrils (section 1.4).

1.3 Elastin

Elastin is the major constituent of mature elastic fibres and is largely responsible for the reversible extensibility (or elastic recoil) of many tissues such as aorta, lung and ligaments. Within the elastic fibre, elastin is found as a polymer surrounded by a

peripheral mantle of microfibrils containing amongst other proteins, the glycoprotein fibrillin (Mecham and Heuser, 1991; Rosenbloom *et al.*, 1993).

Elastin is a highly hydrophobic protein which is synthesised as a soluble molecule known as tropoelastin. This 72kDa protein has an unusual structure, containing alternating hydrophobic and hydrophilic regions (Foster *et al.*, 1973; Bressan *et al.*, 1987; Raju and Anwar, 1987; Indik *et al*, 1987). The hydrophilic regions contain potential crosslinking sites which consist of two lysine residues within a polyalanine sequence. In an α-helical conformation these lysine residues would be orientated on the same side of the helix and would thus be available for the formation of tetra-functional, interchain crosslinks initiated by lysyl oxidase (section 1.4.6). The hydrophobic regions, meanwhile, are believed to form a random coil structure. Together these two regions form the basis of the elastic recoil property of elastin (Mecham and Heuser, 1991).

1.4 Lysyl oxidase

Lysyl oxidase (E.C 1.4.3.13) is a copper amine oxidase (CAO; section 1.4.5) which has been isolated and characterised from a range of vertebrate tissues including human placenta, bovine aorta, porcine skin, rat lung and chick aorta and cartilage (for reviews see Kagan and Trackman, 1991; Kagan, 1994). In mammalian tissue, the mature enzyme has a molecular mass of between 29-34kDa whilst chick aortic lysyl oxidase has a reported molecular mass of 59-61kDa (Kuivaniemi *et al.*, 1984; Kagan *et al.*, 1979; Shackleton and Hulmes, 1990a; Romero-Chapman *et al.*, 1991; Cronshaw *et al.*, 1995; Narayanan *et al.*, 1974; Harris *et al.*, 1974). Evidence also exists for the formation of multimers of lysyl oxidase of up to 100kDa (Kagan *et al.*, 1979).

Four variants of lysyl oxidase have been identified which can be separated by DEAE anion exchange chromatography (Stassen, 1976). The variants have almost identical molecular weights and enzyme activities and they do not appear to be the result of different glycosylation, phosphorylation or disulphide bonding patterns (Kagan *et al.*, 1979; Sullivan and Kagan, 1982; Williams and Kagan, 1985). It has thus been

suggested that the variants are genetically distinct forms of lysyl oxidase (Kagan *et al.* 1979). The recent discovery of a gene encoding for a lysyl oxidase-like (LO-L) protein supports this hypothesis, though the molecular mass of the mature form of LO-L is unknown (Kim *et al.*, 1995).

1.4.1 Purification of lysyl oxidase

Initial attempts to purify lysyl oxidase were hampered by the low solubility and instability of lysyl oxidase in neutral salt buffers (Pinnell and Martin, 1968). Observations that the stability of lysyl oxidase was increased in the presence of urea led to the widespread use of 4-6M urea both for the initial extraction of lysyl oxidase and throughout the purification process (Narayanan *et al.*, 1974). The majority of purification strategies developed for lysyl oxidase have involved some kind of DEAE anion exchange step in combination with collagen or elastin affinity columns (Stassen, 1976; Harris *et al.*, 1974). Additionally, many workers have used a final gel filtration step on Sephacryl S-200 in the presence of urea (Kagan *et al.*, 1979). Purification has also been achieved by Sephacryl S-200 chromatography in the absence of urea in which case lysyl oxidase interacts with the column and can be eluted with urea (Shackleton and Hulmes, 1990a). Other less commonly used purification steps include Cibacron blue dye chromatography, hydroxyapatite chromatography and chromatofocusing (Williams and Kagan, 1985; Almassian *et al.*, 1991; Hans and Tanzer, 1979).

1.4.2 Structure of lysyl oxidase

The cDNA sequence of human placental lysyl oxidase encodes for a protein of 420 amino acids in length comprised of a 21 amino acid signal peptide, an N-terminal propeptide and the 30kDa lysyl oxidase domain (Hamalainen *et al.*, 1991). Three potential N-glycosylation sites have been identified all of which are found within the N-terminal propeptide (Hamalainen *et al.*, 1991; Cronshaw *et al.*, 1995). cDNA sequences are also available for rat and chick aortic lysyl oxidase with overall homologies of 84% and 81% respectively (Trackman *et al.*, 1990; Wu *et al.*, 1992a).

The proteins are most highly conserved within the later (C-terminal) two thirds of the molecule which comprises the sequence for the mature lysyl oxidase protein and contains a number of sequences of interest. These include the sequence His-X_4-Cys-X_5-Cys-X_4-His which is similar to a consensus sequence for metal-binding proteins and two sequences, Tyr-Gly-Tyr-His-Arg-Arg-Phe-Ala-Cys and Tyr-Thr-Gly-His-His-Ala-Tyr, with similarities to certain copper binding sequences (Trackman *et al.*, 1990; Hamalainen *et al.*, 1991). In addition, the first nine N-terminal residues of the mature lysyl oxidase sequence (Asp-Asp-Pro-Tyr-Asn-Pro-Tyr-Lys-Tyr) are 78% homologous with a tyrosine rich region within the proteoglycan fibromodulin (Asp-Asp-Pro-Tyr-Asp-Pro-Tyr-Pro-Tyr) and it has been postulated that this sequence may be important for the collagen binding properties of both these proteins (Cronshaw *et al.*, 1995). Lysyl oxidase is also highly homologous (89%) to the *ras* recision gene (*rrg*) product which has been shown to counteract the transforming activity of *ras*; thus, it is possible that lysyl oxidase also has tumour suppressor activity (Contente *et al.*, 1990; Kenyon *et al.*, 1991).

1.4.3 Biosynthesis and post-translational modifications of lysyl oxidase

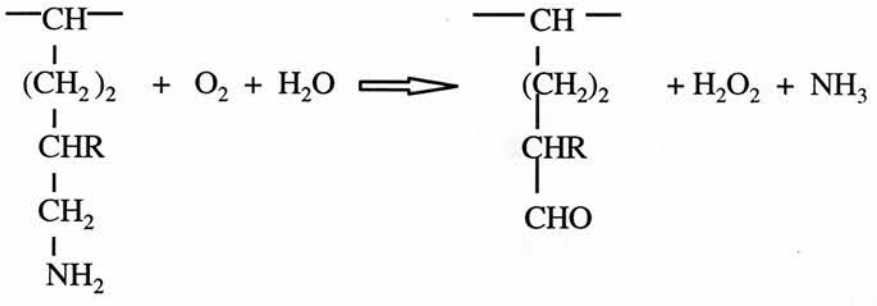
Evidence from cDNA sequencing, immunoprecipitation and pulse chase studies suggests that lysyl oxidase is synthesised as a 46kDa preproenzyme. After cleavage of the signal peptide the protein undergoes N-glycosylation at sites within the propeptide region. The resulting 50kDa proenzyme is secreted and proteolytically processed to the active unglycosylated 29kDa enzyme in the extracellular space (Trackman *et al.*, 1990, 1992; Cronshaw *et al.*, 1995). Recent evidence suggests that the proteinase involved in the processing of lysyl oxidase is procollagen I C-proteinase (BMP-1; Panchenko *et al.*, 1996; section 1.2.2). This implies dual regulation of lysyl oxidase activity and fibril assembly. The function of the propeptide region is still unclear although it is not necessary for secretion, proper folding or activity of the mature enzyme (Kagan *et al.*, 1995).

1.4.4 Localisation of lysyl oxidase within tissues

Lysyl oxidase is found within the extracellular space (Siegel *et al.*, 1978) and also in the cytoplasm of a number of fibroblastic and non-fibroblastic cells (Wakasaki and Ooshima, 1990). Attempts to localise lysyl oxidase at the ultrastructural level by immunogold labelling and electron microscopy gave contradictory results. Kagan *et al.* (1986) localised the enzyme to the interface between amorphous elastin and microfibrils within elastic fibres of calf and rat aorta but were unable to identify lysyl oxidase within collagen fibrils. In contrast, Baccarani-Contri *et al.* (1989) localised lysyl oxidase to collagen fibres in human placental, aortic and skin tissues and found no labelling of elastic fibre in the aorta and poor to no labelling of the elastic fibre in placental and dermal tissue. When labelling did occur it was localised within elastin as opposed to at the interface between elastin and microfibrils. It is possible that these conflicting results are due to the isolation of antigenically different lysyl oxidase species (for example, lysyl oxidase-like protein; section 1.4) from bovine aorta and human placenta, with different substrate specificities (Baccarrani-Contri *et al.*, 1989; Kim *et al.*, 1995). It should also be noted that the anti-human placental lysyl oxidase anti-serum cross-reacts with a 24kDa protein that co-purifies with lysyl oxidase. It is not known whether this protein is a true degradation product of lysyl oxidase or an unrelated protein (Kuivaniemi *et al.*, 1984).

1.4.5 Lysyl oxidase reaction

Copper amine oxidases (CAO) catalyse the oxidative deamination of amines to aldehydes and have a requirement for copper (Wang *et al.*, 1996). The CAO activity of lysyl oxidase converts the ε-amino group on specific lysine and hydroxylysine residues in both collagen and elastin to their corresponding aldehyde (allysine or hydroyallysine; Fig 1.2). The reaction requires molecular oxygen and results in the release of H_2O_2 and NH_3 (Williamson and Kagan, 1986). The exact role of copper is unclear although it is required for the release of the aldehyde from the enzyme complex (Gacheru *et al.*, 1990). Lysyl oxidase also requires a second, carbonyl cofactor and evidence has suggested that it is a quinone (Williamson *et al.*, 1986; Paz



Peptidyl lysine (R=H)
or
hydroxylysine (R=OH)

Allysine (R=H)
or
Hydroxyallysine (R=OH)

Figure 1.2. The reaction catalysed by lysyl oxidase. From Kagan, 1986.

et al., 1991). Until recently, controversy has surrounded the exact nature of this cofactor in lysyl oxidase and in CAOs as a whole (for review see Klinman, 1996). It was previously believed that pyrroloquinoline quinone (PQQ) was the cofactor in human placental (van der Meer and Duine, 1986) and bovine aortic (Williamson *et al.*, 1986) lysyl oxidase although the identification of topaquinone (TPQ) at the active site of a range of other mammalian CAOs suggested that lysyl oxidase also contained this cofactor (Janes *et al.*, 1990, 1992). However, unlike the other CAOs studied, lysyl oxidase does not contain the consensus amino acid sequence for the conversion of tyrosine to TPQ (i.e. Asn-Tyr-Asp/Glu; Mu *et al.*, 1992). Recently, bovine aortic lysyl oxidase was found to contain a unique cofactor at its active site. This cofactor, lysine tyrosylquinone (LTQ) is formed by crosslinking of lysine and tyrosine side chains within lysyl oxidase and is the only example of a quinone cofactor formed by crosslinking of two amino acid side chains (Wang *et al.*, 1996).

1.4.6 Crosslink formation in collagen and elastin

The reaction catalysed by lysyl oxidase initiates a number of spontaneous reactions which result in the formation of crosslinks between individual chains within a collagen monomer (intramolecular) and between adjacent collagen monomers (intermolecular). These cross links can be bi- or tri-functional (for reviews see Last *et al.*, 1990; Eyre *et al.*, 1984). The bi-functional crosslinks can be divided into aldol crosslinks, formed mainly by the condensation of 2 allysines to form the aldol condensation product (ACP; Piez, 1968), and Schiff base crosslinks (Kuboki *et al.*, 1981). Schiff base crosslinks can occur between allysine and the ε-amino group of unoxidised lysine forming dehydrolysinonorleucine, between allysine and hydroxylysine to form dehydrohydroxylysinonorleucine or between hydroxyallysine and hydroxylysine forming dehydrodihydroxylysinonorleucine. Two major tri-functional crosslinks occur in collagen, formed either by reaction of the ACP with histidine to form aldol histidine (Mechanic *et al.*, 1987; Yamauchu *et al.*, 1987) or by reaction of 2 hydroxyallysine residues with either lysine or hydroxylysine to form 3-hydroxypyridinium crosslinks (Fujimoto, 1977; Kagan, 1994).

In elastin, ~85% of the lysine residues are involved in crosslinking (Rosenbloom *et al.*, 1993). Aldol condensation product and dehydrolysinonorleucine crosslinks are found in elastin but since hydroxylation of lysine residues is uncommon in elastin, crosslinks involving hydroxylysine only occur in collagen (Eyre *et al.*, 1984). In contrast, the tetra-functional crosslinks, desmosine and isodesmosine, are unique to elastin and are formed when allysine residues spontaneously condense with other lysine and allysine residues (section 1.3; Thomas *et al.*, 1963). Despite being tetra-functional, these crosslinks are believed to join only two chains of elastin (Rosenbloom *et al.*, 1993).

1.5 Proteoglycans

Proteoglycans are molecules comprised of a protein core covalently linked to one or more glycosaminoglycan (GAG) chains (for reviews see Gallagher, 1989; Kjellan and Lindahl, 1991). Glycosaminoglycans are long, unbranched polysaccharide chains comprised of repeating disaccharide units. In general, the disaccharide contains a derivative of an amino sugar (either D-glucosamine or D-galactosamine) and either hexuronic acid (D-glucuronic or L-iduronic acid) or galactose units. At least one of these sugars is modified with the addition of sulphate or carboxylate groups to give a net negative charge to the molecule. GAG chains can be classified according to the structure of their repeating disaccharide backbone as hyaluronic acid (HA), the galactosaminoglycans chondroitin sulphate (CS) and dermatan sulphate (DS) and the glucosaminoglycans keratan sulphate (KS), heparin and heparan sulphate (HS; Roden, 1980; Wight *et al.*, 1991). Hyaluronic acid, also known as hyaluronate or hyaluronan, is unique amongst GAGs in that it is not covalently bound to a protein core. It is present as extensive anionic polymers and it assumes a stiff, randomly kinked, coil structure which is able to form networks with itself and, via non-covalent interactions, with a number of other matrix macromolecules (for example in cartilage; Rosenberg *et al.*, 1975; Kjellan and Lindahl, 1991).

As well as large variations in the length, composition and number of GAG chains, there are also large variations in the nature of the protein core. Thus, the definition of

a proteoglycan encompasses a large number of molecules with diverse structures, functions and localisation. In general, proteoglycans can be classified in terms of their localisation into extracellular, intracellular and membrane bound proteoglycans (Iozzo and Murdoch, 1996).

1.5.1 Extracellular proteoglycans

The extracellular proteoglycans can be divided into small leucine rich proteoglycans (SLRP) and modular proteoglycans (for review see Iozzo and Murdoch, 1996). The SLRP family consists of the DS/CS proteoglycans decorin, biglycan and epiphycan and the KS proteoglycans fibromodulin and lumican (Krusius and Ruoslahti, 1986; Fisher *et al.*, 1989; Shinomura and Kimata, 1992; Oldberg *et al.*, 1989; Blockberger *et al.*, 1992). As the name suggests these proteins are characterised by a large central domain containing a number of 24 amino acid long, leucine-rich repeats (LRR) which is flanked on either side by highly conserved cysteine-rich regions. The attachment sites for GAG chains are found within this central region in fibromodulin and lumican (Plaas *et al.*, 1990; Blockberger *et al.*, 1992) and within the N-terminal region for decorin, biglycan and epiphycan (Chopra *et al.*, 1985; Fisher *et al.*, 1989; Shinomura and Kimata, 1992; Figure 1.3). Decorin and biglycan also have N-linked glycosylation sites within the leucine-rich repeats and in fibromodulin and lumican the N-terminal region contains sites for tyrosine sulphation (Antonsson *et. al.*, 1991; Blockberger *et al.*, 1992). The SRLPs appear to be multifunctional molecules with potential roles in regulation of collagen fibrillogenesis and the supramolecular organisation of the ECM (Table 1.2; section 1.7), inhibition of cell attachment and regulation of cell growth (Iozzo and Murdoch, 1996). For example, in addition to the role of decorin in fibrillogenesis (section 1.7) it also binds to fibronectin and inhibits fibronectin-mediated cell adhesion (Lewandowska *et al.*, 1987) and binds to and inhibits the activity of the growth factor TGF-β. Additionally, TGF-β stimulates the synthesis of decorin by a number of cell types (Ruoslahti and Yamaguchi, 1991; Figure 1.3).

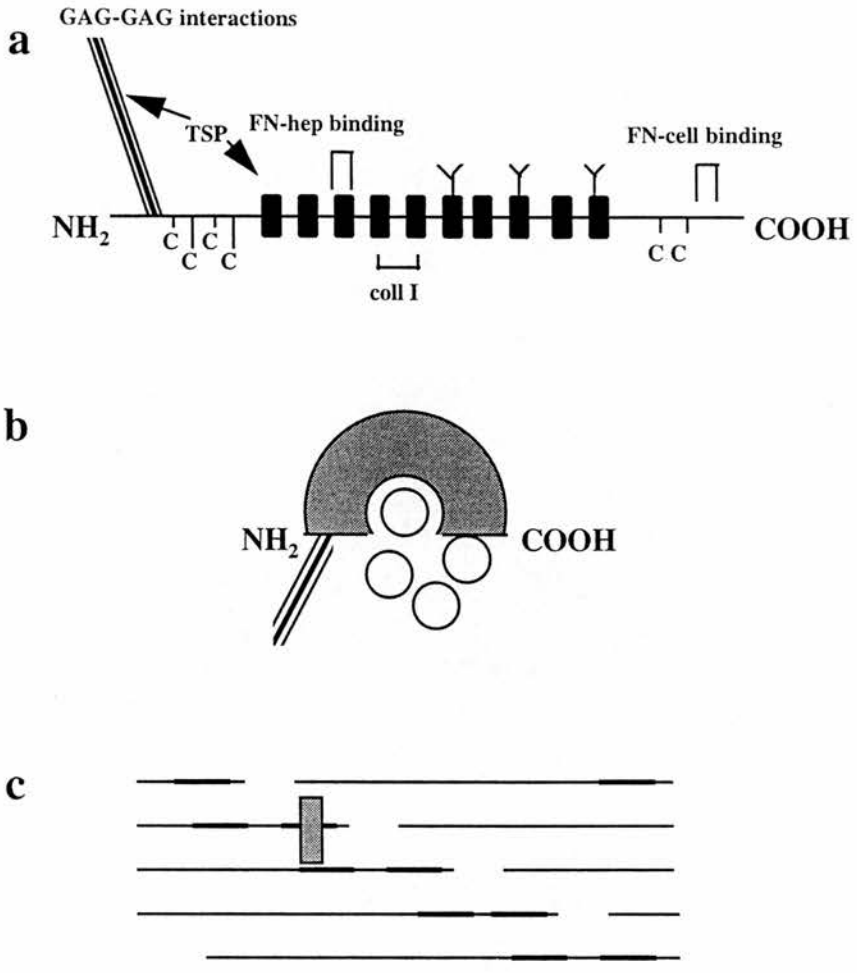


Figure 1.3. Schematic diagram of the structure of decorin and its interaction with collagen monomers and fibrils. (a) Binding domains of decorin; rectangles represent leucine rich repeats , represents GAG chain and Y represents N-glycosylation sites; Putative binding sites for fibronectin (FN), thrombospondin (TSP), collagen I (coll I) and glycosaminoglycans (GAGs) are indicated. C represents cysteine residues. The leucine-rich repeats give rise to the three-dimensional horseshoe structure of decorin seen in cross section in (b). Circles represent collagen monomers. Decorin is believed to interact with two monomers, one within the arch of the horseshoe and another via the C-terminal arm. This would allow the monomers to be correctly orientated in the quarter staggered array within the growing fibril (b,c). From Kresse *et al.*, 1993; Iozzo and Murdoch, 1996; Weber *et al.*, 1996.

The modular proteoglycans have less sequence homology than the SRLPs but are grouped together since they are comprised of various protein modules in a relatively elongated and often highly glycosylated structure. They can be further sub-divided into the hyalectins and the non-hyaluronan binding proteoglycans (Iozzo and Murdoch, 1996). The hyalectin family includes aggrecan, versican, neurocan and brevican and contains an amino terminal domain with hyaluronan binding properties, an extended central domain with GAG binding sites and a carboxyl terminal domain with homology to the selectin family (Doege *et al.*, 1987, 1991; Zimmerman and Ruoslahti, 1989; Rauch *et al.*, 1992; Yamada *et al.*, 1994). The most well studied of the hyalectins is the cartilage proteoglycan, aggrecan. It is able to form large supramolecular aggregates with hyaluronan (section 1.5) through its amino terminal domain and has a large number of GAG chains attached to the central domain. Thus, aggrecan has a high charge density resulting in a large hydrodynamic volume and the ability to withstand compressive forces (Gallagher, 1989). The hyalectins are also believed to function as a bridge between cells and the ECM, with the hyaluronan binding domain interacting with the hyaluronan surface coat of some cells and the lectin-like domain interacting with carbohydrate moieties on various ECM molecules (Iozzo and Murdoch, 1996). Perlecan, agrin and testican make up the non-hyaluronan binding family of proteoglycans (Noonan *et al.*, 1991; Tsen *et al.*, 1995; Alliel *et al.*, 1993). Perlecan is the major HS proteoglycan of basement membranes with potential roles in maintaining a negatively charged barrier, cell adhesion / anti-adhesion and regulation of cell growth by binding to released growth factors and cytokines and sequestering their effect (Iozzo *et al.*, 1994).

1.5.2 Intracellular and membrane bound proteoglycans

The small proteoglycan, serglycin is found intracellularly within secretory vesicles and the core protein contains extended sequences of repeating serine and glycine residues (Avraham *et al.*, 1988; Seldin *et al.*, 1985). These serine-glycine residues represent GAG attachment sites and provide the potential for a highly glycosylated and thus negatively charged molecule. Serglycin is proposed to have roles not only

relating to its polyanionic nature (e.g. reduction of osmotic pressure) but also in binding proteases within vesicles thus modulating their activity (Kresse *et al.*, 1993). Membrane bound proteoglycans also exist which associate covalently with the phospholipid bilayer either via a transmembrane domain within the protein core (e.g. syndecan) or a phosphatidyl inositol membrane anchor, or non-covalently through proteoglycan or GAG cell surface receptors (e.g. hyaluronan; for review see Gallagher, 1989).

1.6 Tyrosine rich acidic matrix protein (TRAMP)

In addition to the four major classes of macromolecule found in the ECM (i.e. collagens, elastin, proteoglycans and glycoproteins), a number of less well characterised, low molecular weight proteins are present. One such protein is Tyrosine-Rich Acidic Matrix Protein (TRAMP) which was originally identified as the major contaminant in preparations of lysyl oxidase from porcine skin (Shackleton and Hulmes, 1990a). Subsequent purification and amino acid sequencing of this protein showed it to be unrelated to lysyl oxidase but identical to bovine 22K ECM protein (Cronshaw *et al.*, 1993; Neame *et al.* 1989). The human equivalent, dermatopontin, was later identified as a contaminant in preparations of proteoglycans from human fibroblast cultures and cDNA sequencing showed it to have 96% homology to the bovine form (Superti-Furga *et al.*, 1993).

1.6.1 Structure of TRAMP

The amino acid sequence of TRAMP is characterised by a high percentage of tyrosine residues (9.7%), no N-glycosylation sites and three homologous domains characterised by the presence of positively charged residues and a central hexapeptide motif (i.e. Asp-Arg-Glx-Trp-Asn/Gln/Lys-Phe/Tyr; Cronshaw *et al.*, 1993). Five disulphide bonds have been identified between Cys^{32}-Cys^{59}, Cys^{121}-Cys^{178}, Cys^{72}-Cys^{114}, Cys^{88}-Cys^{115} and Cys^{125}-Cys^{171}. It has been suggested that the arrangement of disulphide bridges within TRAMP would place one of the hexapeptide repeats in each of three loop structures which would allow interactions between these motifs

and other ECM molecules (Neame *et al.*, 1989). Additionally, an 18 amino acid, N-terminal signal sequence is present within the cDNA sequence of dermatopontin (Superti-Furga *et al.*, 1993). Analysis for hydrophobicity showed the protein to be very hydrophilic except for two regions of strong hydrophobicity around residues 40 and 90. Secondary structure prediction using the method of Chou and Fasman showed no overall tendency for either β-sheet or α-helix, although this has yet to be confirmed by circular dichroism (Neame *et al.*, 1989).

1.6.2 Tyrosine sulphation of TRAMP

Five charge variants of TRAMP have been identified, separable by Mono Q anion exchange FPLC (Cronshaw *et al.*, 1993). Their pI values vary from 4.43 to 4.07 and their molecular masses from 22,119 to 22,352 daltons. Evidence suggests that the variants are the result of different patterns of tyrosine sulphation within the protein. Tyrosines 5, 15, 148, 149 and 176 fit the empirically determined rules for tyrosine sulphation involving the proximity of acidic residues and relative paucity of basic residues, hydrophobic residues and cysteine (Hortin *et al.*, 1986; Cronshaw *et al.*, 1993). In addition, TRAMP can be visualised on SDS-PAGE gels by Alcian blue staining and this staining, relative to Coomassie staining is decreased if the protein is subjected to acid hydrolysis or sulphatase treatment (Cronshaw *et al.*, 1993). Finally, ^{35}S-labelled TRAMP is synthesised by human foreskin fibroblasts cultured in the presence of [^{35}S]-sulphate and alkaline hydrolysis followed by amino acid analysis has confirmed that the radiolabelled sulphate is incorporated into sulfotyrosine (Forbes *et al.*, 1994).

Tyrosine sulphation is a widespread post-translational modification and in addition to TRAMP, a number of other ECM proteins are modified in this way. These include fibromodulin, procollagens III and V, fibronectin, vitronectin, osteopontin (BSPII) and entactin (Antonsson *et al.*, 1991; Jukkola *et al.*, 1986; Fessler *et al.*, 1986; Paul and Hynes, 1984; Jenne *et al.*, 1989; Ecarot-Charrier *et al.*, 1989; Paulsson *et al.*, 1985). The significance of tyrosine sulphation remains to be elucidated. Since the majority of tyrosine sulphated proteins identified to date are secreted, it has been

postulated that tyrosine sulphation may play a role in the secretory mechanism (Huttner, 1988). However, inhibition of tyrosine sulphation by chlorate had no effect on the synthesis or secretion of procollagen III from human skin fibroblasts (Jukkola *et al.*, 1990). Alternatively, it has been suggested that the increase in negative charge which accompanies incorporation of sulphate into a protein may influence subsequent interactions of that protein. For example, partial removal of sulphate groups from fibronectin results in a decrease in the fibrin binding activity (Suiko and Lui, 1988).

1.6.3 Tissue distribution of TRAMP

Western blot analysis of TRAMP show it to be a widely distributed protein in porcine and murine tissue. Positive results were observed for skin, cartilage, long bone, calvaria, skeletal muscle, lung, heart and kidney with no detectable TRAMP in liver, brain or spleen (Forbes *et al.* 1994). This is consistent with the expression of dermatopontin in human tissues analysed by Northern blotting, where expression was also observed in pancreas (Superti-Furga *et al.* 1993). Dermatopontin has been further localised, with antibodies to the bovine form of TRAMP, both around collagen fibres and unexpectedly within dermal endothelial cells in human skin biopsies (Okamoto *et al.*, 1996).

1.6.4 The biological function of TRAMP

The biological function of TRAMP is still unclear, although evidence to date suggests a number of possibilities:-

1.6.4.1 Attachment of cells to the extracellular matrix.

The bovine form of TRAMP (22K) has been shown to promote cell adhesion of mouse Balb/c 3T3 cells, human and bovine dermal fibroblasts, human Platt neuroblastoma cells and F11 hybrid neuronal cells, when absorbed onto tissue culture cluster dishes (Lewandowska *et al.*, 1991). Approximately 25% of 3T3 cells attach

stably, representing 35-40% of the levels of plasma fibronectin (pFN) mediated attachment. Phase contrast and scanning electron microscopy showed that cells undergo attachment and spreading on 22K substrata but no more extensive cytoskeletal reorganisation is evident. It was postulated that the cell surface receptor for 22K was an integrin since cell attachment to the substrata was inhibited by the addition of a Gly-Arg-Gly-Asp-Ser-Pro-Cys (GRGDSPC) peptide (section 1.8). Also, 22K contains the putative integrin binding site RGAT (Hautanen *et al.*, 1989; Ruoslahti, 1987; Ruoslahti and Pierschbacher, 1987; Yamada and Kennedy, 1987). Since pre-treatment of the cells with chondroitinase ABC had no effect on cell attachment, the possibility that the receptor was a cell surface DS proteoglycan seems unlikely. Cell attachment of 22K was inhibited by decorin (Lewandowska *et al.*, 1991; section 1.5.1).

1.6.4.2 Regulation of collagen I fibril assembly

Macbeath *et al.* (1993) showed that TRAMP accelerates collagen I fibril formation *in vitro* using the 'warm start' technique (Holmes *et al.*, 1986; section 1.7.1). The effect was characterised by a decrease in the lag phase and an increase in both the growth rate and the final turbidity (Figure 1.4). Acceleration was still observed at sub-stiochiometric ratios of TRAMP to collagen (i.e. molar ratios of 1:20) suggesting the possibility that TRAMP acts on the early nucleation phase of fibrillogenesis. It was also observed that TRAMP partially reversed the inhibitory effects of increased ionic strength and urea but not of glucose. Since glucose is believed to inhibit fibrillogenesis by interacting with the non-helical telopeptides of collagen it is possible that TRAMP also interacts with them. However, TRAMP also accelerates fibrillogenesis of pepsinised collagen I and thus it may interact with collagen monomers at more than one site. The presence of TRAMP stabilises collagen fibrils against dissociation when the temperature is dropped to 4°C and also results in a decrease in fibril diameter. All TRAMP variants are equally effective at accelerating fibril formation (Macbeath *et al.*, 1993).

The bovine form of TRAMP has recently been shown to interact with the protein core of the dermatan sulphate proteoglycan, decorin (section 1.5.1; Okamoto *et al.* 1996). The interaction is disrupted by guanidine hydrochloride but not by cleavage of the disulphide bonds present in either decorin or 22K. The significance of this interaction is unclear but since decorin is known to inhibit collagen fibrillogenesis *in vitro* (Vogel *et al.,* 1984; section 1.7.3) it is plausible that TRAMP and decorin work together to regulate the deposition of collagen in the ECM. Additionally, since TRAMP appears to have cell adhesion activity it may also have a role in the cellular control of fibril deposition (section 1.7.4).

1.6.4.3 Crosslinking of collagen

TRAMP contains the putative topaquinone incorporation consensus sequence Asn-Tyr-Asp, which is associated with a number of copper dependent amine oxidases (Mu *et al.*, 1992; section 1.4.5). Additionally, preliminary investigations showed that after incubation of TRAMP with [4,5-^{3}H] lysine labelled collagen I at 37°C overnight followed by SDS-PAGE analysis, an increase in the $\beta_{1,2}$ components was observed (Forbes, unpublished observations). Since the $\beta_{1,2}$ components correspond to dimers of α chains this suggested that intramolecular crosslinks were formed. These observations together with the ability of TRAMP to stabilise collagen fibrils against low temperature dissociation (Macbeath *et al.*, 1993; section 1.6.4.2) have led to the suggestion that TRAMP may act as a collagen specific lysyl oxidase.

1.7 Collagen fibril assembly

Matrix assembly occurs not only during growth and development of an organism but also during repair and regeneration (for review see Birk *et al.*, 1991). The assembly of the matrix is a complex process involving many interactions between adjacent matrix molecules and between matrix molecules and the cell. Fibrillar collagens are the major components of most connective tissues and thus the physical properties of tissues are determined to a large extent by the three-dimensional organisation of collagen fibrils. Since the various collagen types have very different supramolecular

structures, matrix assembly is dictated by the tissue specific expression of unique collagen types. However, tissues which produce the same collagen type can have very different structures and thus additional controls on matrix assembly must occur. The assembly and correct spatial arrangement of collagen fibrils is affected by other matrix components such as proteoglycans. In addition, the assembly process is controlled at the cellular level via integrin and non-integrin receptors. Since the major step in matrix assembly is formation of collagen fibrils, the following section will concentrate on collagen fibrillogenesis and in particular the assembly of collagen I fibrils (for review see Kadler *et al.*, 1996).

1.7.1 Collagen fibril assembly *in vitro*

Solutions of extracted collagen in neutral buffers have long been known to aggregate spontaneously into fibrils when the temperature is raised (Wood and Keech, 1960). The characteristic turbidity-time curve obtained by spectrophotometric analysis of the assembly process is triphasic. First there is a lag phase due to the time it takes to produce fibrils of sufficient size to be detected by scattering of light. This is followed by a period of rapid growth and finally, when fibril assembly is complete, a plateau region (Figure 1.4; Gross and Kirk, 1958; Williams *et al.*, 1978).

Until recently, the most common method used to initiate fibril formation was the 'cold-start' technique whereby cold acidic solutions of collagen are first mixed with an equal volume of double strength neutralising buffer before raising the temperature (Williams *et al.*, 1978). To eliminate problems caused by precipitation after neutralisation in cold conditions, the 'warm-start' technique was developed (Helseth and Veis, 1981). In this procedure, both collagen and neutralising solutions are first warmed to the desired temperature and are then mixed to initiate fibril assembly (Holmes *et al.*, 1986). However, difficulties also arise from this method due to variations in assembly rate caused by mixing and stirring currents. Thus, both these methods require strict controls for reproducible results to be obtained. An alternative method to study fibril assembly and growth was described by Kadler *et al.* (1987). In this procedure fibril assembly is initiated by specific enzymatic cleavage of the

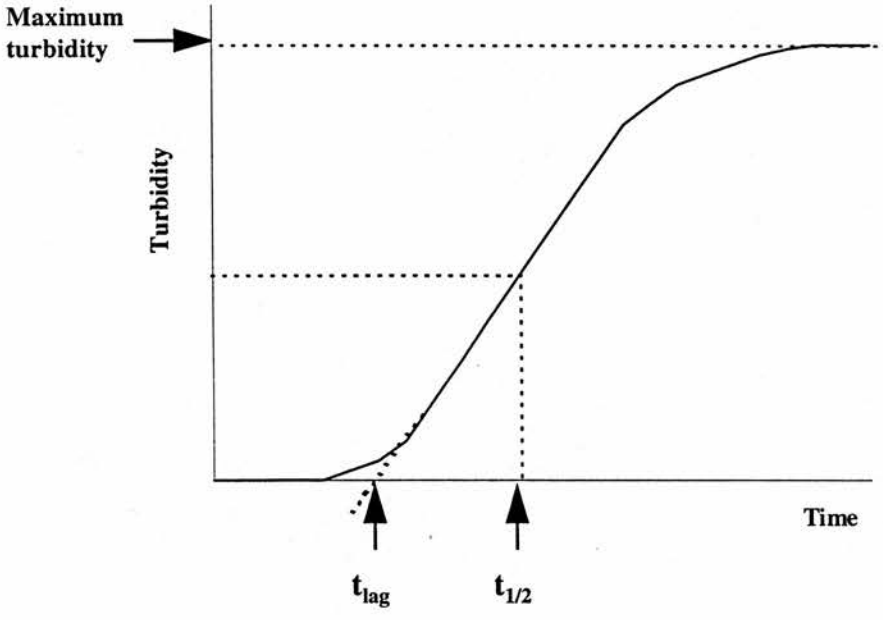


Figure 1.4. Typical turbidity-time curve for collagen I fibril assembly *in vitro*. The lag time (t_{lag}), maximum turbidity and time to half maximum turbidity ($t_{1/2}$) are marked by arrows. Additionally, the rate of growth during the growth phase can be calculated from the gradient of the line.

C-propeptide from pC-collagen (a soluble precursor, lacking the N-propeptide) using C-proteinase/BMP-1.

1.7.2 Possible mechanisms of collagen fibril assembly

The ability of fibril-forming collagens to self-assemble *in vitro* suggests that the assembly process is controlled largely by the amino acid sequence of collagen and the distribution of polar and hydrophobic residues (Veis and George, 1994; Prockop and Kivirrikko, 1995). Evidence also exists for initiation of fibril assembly by conformational changes within the monomer which 'perfect' the triple helix (George and Veis, 1990, 1991). The mechanism of assembly remains unclear although two main hypotheses exist to explain the kinetics observed *in vitro*. One theory is based on a nucleation-growth process where the rate-limiting step is the formation of an initial aggregate or nucleus onto which monomers can rapidly add to form the final D-periodic fibril. The second theory suggests an intermediate-assembly process whereby small intermediate aggregates form during the lag phase which can then add to each other to form a fibril (Veis and George, 1994). Electron microscopy and light scattering studies on collagen solutions at various stages of the assembly process have led to contradictory observations offering support for both these theories. Some workers have identified 4D staggered dimers and trimers early in the lag phase which then appear to aggregate linearly (Trelstad and Hayashi, 1976; Silver and Trelstad, 1979; Gelman and Piez, 1980; Silver, 1981; Kadler and Chapman, 1985; Ward *et al.*, 1986). Other workers have been unable to see such species and thus observe the long thin intermediate subassemblies or 'early' fibrils as the first formed aggregates (Gelman *et al.*, 1979; Payne *et al.*, 1986; Na *et al.*, 1986). The conflicting results may be explained by the observation that the method used to initiate fibril formation affects the route of assembly and thus the nature of aggregates formed (Holmes *et al.*, 1986). Also, identification of the first formed aggregates in the pathway is hampered by the low concentrations of such species present and by the production of artefactual aggregates (Kadler *et al.*, 1996).

Further understanding of the mechanism of assembly can be gained by studying the differences in kinetics observed from collagen solutions obtained by different purification methods. The presence of a high percentage of crosslinked oligomers results in a decrease in the lag phase compared to preparations of purely monomeric collagen suggesting that the oligomers already contain the nucleation centres and thus supporting a nucleation growth process (Na *et al.*, 1986). Pepsin-treatment of collagen increases the lag phase suggesting a role for the N-and C-non-helical telopeptide regions in the nucleation phase (Helseth and Veis, 1981).

As mentioned above, 'early' fibrils have been identified during the initial stages of fibril assembly and in particular towards the end of the lag phase. These are usually banded (depending on the initiation procedure) and between 1 and 20μm in length (Holmes *et al.*, 1986). The development of the cell free system for studying fibril formation (section 1.7.1) allowed for further study of these 'early fibrils' since at low temperatures (29-32°C) the diameters of the fibrils are sufficiently large to be visualised by dark field light microscopy and the growth of the fibril can be monitored by time lapse photography (Kadler *et al.*, 1987, 1988). The earliest aggregates observed by this method are needle-like with a pointed, paraboloidal α-tip and a blunt end (Kadler *et al.*, 1990; Holmes *et al.*, 1992). Growth occurs exclusively from the α-tip unless the temperature and concentration of pC-collagen are raised, at which point growth also occurs from the blunt end producing a β-tip. It is thus suggested that *in vivo* growth occurs first at the α-tip and later at the blunt end (Kadler *et al.*, 1990). The shape of the tips appears to be set up early during growth since it is maintained as growth proceeds (Holmes *et al.*, 1992). Another interesting observation is that the ratio of C-proteinase to pC-collagen affects the shape of the tip and symmetry of the fibrils with finer tips and more assymetric fibrils being produced at low ratios (Holmes *et al.*, 1996). It is unclear how fibril growth occurs although recently two computer generated models have been devised to try and explain the observation from the *de novo* system. The first is a helical model of nucleation and propagation (Silver *et al.*, 1992) and the second is based on diffusion limited aggregation of collagen monomers (Parkinson *et al.*, 1995). The former requires very specific growth rules whilst the latter suggests a more random process. Recent

observations on fibril segments from chick tendon at various stages of development, suggest that whilst addition of individual monomers onto the growing fibril may account for the initial assembly of fibrils, maturation of fibrils involves fusion of fibril intermediates. (Birk *et al.*, 1995, 1996, 1997).

1.7.3 Parameters affecting the kinetics of fibril assembly

In vitro the rate of fibril assembly is not only dependent on the method used to initiate the process but also on the composition of the neutralising buffer. The ionic strength, pH, nature of the micro-ion and dielectric constant all affect the kinetics and the morphology of fibrils formed (Hayashi and Nagai, 1973a,b; Williams *et al.*, 1978; Pogany *et al.*, 1994). In addition, the kinetics are sensitive not only to the starting concentration of collagen but also its composition, source and the method used to purify it (Veis and George, 1994). For example, the presence of even small traces of aggregates can substantially decrease the lag phase (Na *et al.*, 1986). The kinetics are also highly temperature-dependent with an optimal temperature for fibril assembly of between 34 and 36°C (Williams *et al.*, 1978). The kinetics even appear to be sensitive to the shape of the reaction vessel used and the mode of heating (Veis and George, 1994).

Both glycosaminoglycans and proteoglycans have been shown to alter the kinetics of fibrillogenesis *in vitro*. In general, GAGs which interact most strongly with collagen fibrils have the greatest effect on fibrillogenesis. In particular, dermatan sulphate, heparan sulphate and heparin accelerate the assembly process whilst chondroitin sulphate, and keratan sulphate have no effect (Scott, 1988). A number of small leucine-rich proteoglycans have the reverse effect on fibril assembly (Table 1.2). Decorin, lumican and fibromodulin bind collagen and inhibit the assembly of fibrils (Vogel *et al.*, 1984; Scott and Haigh, 1985; Rada *et al.*, 1993; Hedbom and Heinegard, 1989). In contrast, another member of this family, biglycan, has no effect on fibrillogenesis and only binds to collagen at low phosphate concentrations. It may be that unlike decorin, fibromodulin and lumican which interact with collagen via regions on their protein core, biglycan interacts via its glycosaminoglycan chains

ECM Component	Binding to collagen I monomers	Binding to collagen I fibrils	Nature of Interaction	Effect on fibril assembly	Effect on fibril diameter	Other interactions	References
Decorin	✓ (2 sites)	✓ d, e bands	PC (LRR 4-5)	↓	↓	collagens II, VI, XII, TGF-β, FN, TSP, 22K ECM protein	Hedbom and Heinegard, 1989; Vogel *et al.*, 1984; Svensson *et al.*, 1995; Schonherr *et al.*, 1995; Vogel and Trotter, 1987; Bidanset *et al.*, 1992; Font *et al.*, 1993, 1996; Schmidt *et al.*, 1987; Hildebrand *et al.*, 1994; Winnemoller *et al.*, 1992; Okamato *et al.*, 1996
Biglycan	?	only in low $[PO_4]$	CS/DS chain(s)	none	?	TGF-β	Pogany *et al.*, 1994; Brown and Vogel, 1989; Hildebrand *et al.*, 1994
Lumican	?	✓ a, c bands	PC	↓	↓	?	Rada *et al.*, 1993
Fibromodulin	✓	✓	PC	↓	↓	collagens II, XII, TGF-β	Hedbom and Heinegard, 1989,1993; Font *et al.*, 1996; Hildebrand *et al.*, 1994
Epiphycan	?	?	-	?	?	?	Shinomura and Kimata, 1992

Table 1.2. Summary of interactions of small leucine-rich proteoglycans with collagen I and other ECM components.
↓ = decrease, ↑ = increase, ✓ = binding, X = no binding, ? = unknown. FN = fibronectin, TSP = thrombospondin, TGF-β = transforming growth factor-β, LRR = leucine-rich repeat, PC = protein core, CS/DS = chondroitin sulphate / dermatan sulphate.

ECM Component	Binding to monomers	Binding to fibrils	Effect on fibril formation	Effect on fibril diameter	References
Fibronectin	✓	✓	↓	?	Kleinman *et al.*, 1981
Link protein	✓	✓	↓	?	Chandrasekhar *et al.*, 1983
Osteopontin (BSPII)	?	✓	↑	?	Fujisawa and Kuboki, 1992
Osteocalcin (BGP)	?	✓	?	?	Fujisawa and Kuboki, 1992
Osteonectin (SPARC)	?	✓ and X	?	?	Fujisawa and Kuboki, 1992
Dentin Phosphoryn	?	✓	↓	none	Cocking-Johnson and Sauk, 1983
Vitronectin	✓	✓	?	?	Gebb *et al.*, 1986
Myelin ass. glycoprotein (MAGP)	✓	✓	↓	↑	Probstmeier *et al.*, 1992
Thrombospondin	?	✓	?	?	Cockburn and Barnes, 1991
TRAMP	?	✓	↑	↓	Macbeath *et al.*, 1993
Flexilin	?	✓	?	?	Lethias *et al.*, 1996

Table 1.3. Summary of interactions of non-proteoglycan components of the ECM with collagen I.
↓ = decrease, ↑ = increase, ✓ = binding, X = no binding, ? = unknown.

(Pogany *et al.*, 1994). It has been observed that both fibromodulin and decorin bind to collagen monomers at higher molar ratios than to collagen fibrils, suggesting roles in early nucleation phases of fibril assembly (Hedbom and Heinegard, 1993).

A number of other non-collagenous proteins are known to affect the rate of fibril assembly *in vitro*. Inhibitory effects have been observed for fibronectin, dentin phosphoryn and myelin-associated glycoprotein (MAGP), whilst osteopontin and TRAMP (section 1.6.4.2) accelerate the assembly process (Kleinman *et al.*, 1981; Cocking-Johnson and Sauk, 1983; Probstmeier *et al.*, 1992; Macbeath *et al.*, 1993; Fujisawa and Kuboki, 1992). A number of other components of the ECM including vitronectin and flexilin have been shown to bind to collagen but their effect, if any on fibril assembly has yet to be determined (Gebb *et al.*, 1986; Lethias *et al.*, 1996; Table 1.3).

1.7.4 Control of Fibril Diameter and Growth and Supramolecular Organisation

The diameters of collagen I fibrils vary within the same tissues, among different tissues and during development. Thus, strict tissue specific controls on fibril diameter must exist *in vivo*. A number of factors which appear to regulate fibril diameter, growth and the supramolecular organisation of fibrils within tissues are discussed below.

Immunoelectron microscopy has localised the N- and C-propeptides on or near collagen fibrils in a number of tissues (Fleischmajer *et al.*, 1983, 1985, 1987a, 1987b). It is unclear whether this is due to surface association of cleaved propeptides or to pN- and pC-collagen intermediates. However, changes in fibril diameter during development of chick embryos are accompanied by changes in the pN/pC-collagen ratio (Fleischmajer *et al.*, 1985). Also, the order of cleavage of the propeptides affects fibril diameter, with thinner fibrils being formed if the C-propeptide is cleaved first (Fleischmajer *et al.*, 1987b). It has thus been suggested that the N-propeptide is not cleaved before fibril assembly so that pN-collagen is incorporated into the growing fibril. The N-propeptide is constrained to the surface of the growing fibril and eventually becomes tightly packed, limiting the diameter of the

fibril. Cleavage of the N-propeptide must occur before the fibril can continue to grow (Kadler *et al.,* 1996). This theory also fits in with the observation *in vivo* that fibrils have preferred diameters which are multiples of 11nm (Parry and Craig, 1979; Hulmes, 1983). Additionally, the persistence of the procollagen N-propeptide appears to affect fibril morphology (Hulmes *et al.*, 1989; Watson *et al.*, 1992)

Electron microscopy studies have also localised a number of proteoglycans, including decorin and lumican, to collagen fibrils *in vivo* (Scott, 1980; Scott and Orford, 1981: Scott and Haigh, 1985). The type of interaction varies from tissue to tissue. In the corneal stroma and developing tendon, proteoglycans are interfibrillar, bridging one fibril to another and thus maintaining a stable orientation between neighbouring collagen fibrils (Doane *et al.*, 1992; Scott, 1993). There is also evidence that proteoglycans can be intrafibrillar, for example in the collagen II-rich fibrils of costal cartilage (Scott, 1985). It has been suggested that they may have a role in the aggregation of 'early fibrils' into larger mature fibrils (Birk *et al.*, 1995). Proteoglycans have also been shown to be surface-associated, either partly or completely encircling the fibril and with specific binding sites. In this case, it is believed that their role is to limit fibril diameter and/or growth (Scott, 1993). Additionally, certain proteoglycans are able to bind FACIT collagens and collagen VI as well as fibrillar collagens. They may therefore mediate the interactions between non-fibrillar collagens and fibrillar collagens (Font *et al.*, 1993, 1996; Keene *et al.*, 1996). Recent studies on the three-dimensional structure of decorin has suggested a role in the correct positioning of collagen molecules within the staggered conformation of the fibril. Rotary shadowing and molecular modelling studies show decorin to be horseshoe shaped and it is postulated that it binds to one collagen monomer within the recess and to a second, adjacent monomer via one of the arms of the horseshoe (Figure 1.3; Weber *et al.*, 1996; Scott, 1996). The importance of proteoglycans in the regulation of collagen fibril growth was recently highlighted by observations from mice disrupted at the decorin gene locus. The skin from these mice was fragile and had reduced tensile strength. In addition, the collagen fibrils were more loosely packed compared to the skin from normal mice and had variations in diameters along their shafts (Danielson *et al.*, 1997).

Most fibrillar collagens are now believed to be heterotypic (Birk and Lisenmayer, 1994). One reason for this is to control fibril diameter. Both collagen III and pN-collagen III are found associated with type I collagen fibrils in certain tissues (Fleischmajer *et al.*, 1981, 1983, 1990). pN-collagen III has been shown to form true copolymers with collagen I after enzymatic cleavage of precursor forms. The fibrils formed are thinner than fibrils containing collagen I only and the rate of formation of fibrils is slower. Thus, pN-collagen III may regulate fibril diameter by coating the surface of the fibril thereby preventing lateral growth but allowing growth from tips to proceed (Romanic *et al.*, 1991). Recently, the importance of collagen III for collagen I fibrillogenesis was demonstrated by gene knockout experiments. Inactivation of the COL3A1 gene in mice resulted in the reduction or absence of collagen fibrils in many tissues and when present, fibrils had highly variable diameters (Liu *et al.*, 1997). The diameters of collagen I fibrils also appear to be regulated by the presence of collagen V. The connective tissue of the corneal stroma is mostly comprised of collagen I and also contains relatively high amounts of collagen V. In addition, collagen fibrils within the cornea have a very narrow range of small-diameters (25nm) to allow for optical transparency. Thus, a relationship between fibril diameter and collagen V was suggested. Collagens I and V have since been shown to form heterotypic fibrils and fibrils formed *in vitro* from mixtures of these two collagen types have smaller diameters than those formed with collagen I alone (Birk *et al.* 1986a, 1990). More recently, Marchant and co-workers (1996) showed that when the levels of collagen V secreted by chick corneal cells were reduced using a dominant negative retroviral strategy, the fibrils produced had larger diameters and a broader size distribution. Members of the FACIT family of collagens also appear to regulate fibril diameter. Collagen II forms heterotypic fibrils with collagens IX and XI (Mendler *et al.*, 1989). Collagen XI forms a cylindrical core around which molecules of collagen II are arranged. Collagen IX is found exclusively at the surface of the fibril and again may act not only to regulate fibril diameter but also as a bridge between neighbouring collagen fibrils (Wu *et al.*, 1992).

In addition to various extracellular components, the cell is also believed to play a role in the regulation of collagen fibril growth and their organisation into supramolecular

aggregates. Birk *et al.* (1986b) observed a number of extracellular compartments within tendon formed by invaginations of the fibroblast plasma membrane. These compartments could be divided into narrow recesses containing a single collagen fibril and larger indentations enveloping fibril bundles. Further investigation led to the suggestion that the initial events of fibril assembly, such as cleavage of the propeptide and assembly of 'early fibrils', take place in the narrow recesses under tight cellular control. The recesses then fuse to allow individual fibrils to fuse laterally and/or longitudinally forming longer/thicker fibrils. Further fusion of these larger recesses would then allow association of fibrils into bundles (Birk *et al.*, 1989). In addition to this, a further level of cellular control is suggested by the observation that vacuoles within cultured fibroblasts and chondrocytes contain segment-long-spacing (SLS) crystallites (Bruns *et al.*, 1979). Thus, the initial stages of fibrillogenesis may take place intracellularly.

1.8 Cell-Matrix Interactions

Interactions between the ECM and the cell occur via a number of cell surface adhesion receptors which include the integrin family (Newham and Humphries, 1996), membrane associated proteoglycans (section 1.5.2; Gallagher, 1989) and the elastin receptor, which not only binds elastin but also laminin and collagen IV (Hinek, 1996). Additionally, the recently discovered transmembrane collagens may also play a role in cell-matrix communication (section 1.2.1; Hopkinson and Jones, 1996).

Integrins are heterodimers, composed of α and β subunits. Both the α and β subunits have large extracellular domains, a single transmembrane domain and a short cytoplasmic domain. The extracellular domain contains binding sites for a number of ECM ligands including fibronectin, tenascin, thrombospondin, vitronectin, bone sialoprotein and various collagens (for reviews see Ruoslahti, 1991; Newham and Humphries, 1996). The integrin recognition sites within these molecules are generally short peptide sequences (e.g. Arg-Gly-Asp) which are presented in extended loops containing β turns (Humphries, 1990). Binding of ligands activates

cell signalling cascades within the cell via Focal Adhesion Kinase (FAK; Richardson and Parsons, 1995). In addition to this 'outside-in' signalling , integrin activity can also be modulated from inside the cell (inside-out signalling; O'Toole *et al.*, 1994). Thus, regulation of the cell by the surrounding matrix and of the matrix by the cell appears to be a constantly changing subtle process (Newham and Humphries, 1996).

1.9 Aims of project

The initial aim of the project was to produce highly purified, active TRAMP completely free from contaminating lysyl oxidase. Once purified the interactions of TRAMP with collagen I monomers and fibrils could be studied using ELISA, fibrillogenesis and co-sedimentation assays. These methods could also be used to study the combined effect of TRAMP and decorin on collagen I fibril formation *in vitro* and the effect of decorin on the interactions of TRAMP with collagen fibrils and monomers. An additional aim was to identify binding sites for TRAMP on collagen fibrils using electron microscopy and immunogold labelling. Further aims of the project were to investigate the possible amine oxidase activity of TRAMP using benzylamine, elastin and collagen substrates. Finally, we hoped to study in more detail the distribution of TRAMP in murine tissues by immunohistochemistry and western blotting.

Chapter 2

Materials And Methods

2.1 Lysyl oxidase ultrafiltration assay

2.1.1 Preparation of [^{3}H] elastin substrate

Lathyritic [4,5-^{3}H] lysine labelled elastin was prepared as described previously (Siegel *et al.*, 1970; Kagan *et al.*, 1979).

2.1.1.1 Materials

Seventeen day old chick embryos were supplied by Ross Breeders, Newbridge, Midlothian. MEM Select-amine kits were from GIBCO, Paisley, Strathclyde. β-APN (fumarate salt) was from Sigma Chemicals Co., Poole, Dorset. Unless otherwise stated, all other reagents were from BDH, Poole, Dorset.

2.1.1.2 Procedure

Culture media and flasks were sterilised by filtration through 0.22μm filters and autoclaving respectively. Thoracic arteries from 10 dozen, 17-day old chick embryos were dissected and placed into Petri dishes containing MEM Select-amine incubation medium with 50 units/ml penicillin and 50μg/ml streptomycin (GIBCO). This medium lacked lysine but was supplemented with 50mg/l each of glycine, alanine, valine, proline and ascorbate to increase yields of elastin (Shackleton and Hulmes., 1990b). Fifty mg/l of the lathyrogen, β-aminopropionitrile (β-APN) was also added to inhibit endogenous lysyl oxidase activity and the pH was adjusted to 7.4. After washing in 2 changes of this media the arteries were placed in 300ml fresh medium in a Nalgene Erlenmayer flask, which was gassed with 5% CO_2/95% air for 1 minute, sealed tightly and incubated at 37°C in a model G25 incubator shaker (New Brunswick Scientific) for 1 hour to deplete lysine. Arteries were transferred to fresh medium containing 15μCi/ml [4,5^{3}H]-lysine, gassed again and incubated overnight at 37°C. The incubation medium was discarded and the arteries were washed in several changes of cold distilled water. To extract components other than elastin, the arteries were homogenised in a small volume of 0.15M NaCl in a glass homogeniser,

transferred to a centrifuge tube and topped up to 50ml with 0.15M NaCl. The homogenate was then centrifuged at 10,000*g* for 5 minutes at 4°C. The supernatant was discarded and the salt extraction step repeated. To inactivate endogenous lysyl oxidase the pellet was homogenised in 1M hydrochloric acid and centrifuged as above (Kagan *et al.*, 1979). This step was repeated once. The pellet was then homogenised in assay buffer (0.1M sodium borate, 0.15M NaCl, pH 8.0) and again centrifuged as above. After repeating this step once more, the pellet was resuspended in assay buffer to give a final concentration of labelled elastin of 300,000dpm per 100µl. The elastin substrate was stored at -30°C and thawed and re-homogenised just prior to use.

2.1.2 Ultrafiltration assay

Lysyl oxidase activity was measured using a modification of the method of Pinnell and Martin (1968). Active lysyl oxidase will convert the ε-amino groups at the 6 position of [4,5-^{3}H] labelled lysine residues on elastin to allysine (Figure 1.2). Allysine then undergoes keto-enol tautomerism resulting in the exchange of a tritium atom from the 5 position to the aqueous environment. The tritiated water produced is then isolated by ultrafiltration through Millipore MC filter units with a 5kDa cut off. Use of a β-APN control ensures specificity of the assay (Shackleton and Hulmes, 1990b).

2.1.2.1 Procedure

A total of 100µl of freshly homogenised elastin substrate was added to a 1.5ml eppendorf tube and diluted with 700µl assay buffer. One hundred µl of the sample to be assayed was then added, mixed by vortexing and incubated at 37°C for 20 hours. The reaction was stopped by placing tubes on ice and 100µl of 50% (w/v) trichloroacetic acid was added to precipitate high molecular weight, tritium labelled protein. After incubating on ice for 15 minutes, the tubes were centrifuged at 15,600*g* for 5 minutes. A total of 420µl of the supernatant was removed, placed in a Millipore

Ultrafree-MC filter unit (5kDa cut off) and centrifuged at 5,000*g* for 120 minutes at 4°C in a Beckman JA18.1 rotor. After this, 300µl of the filtrate was removed, added to 2.7ml Ultima Gold liquid scintillation cocktail (Packard) and ^{3}H cpm were determined using a Packard 1900 CA liquid scintillation analyzer. For each sample assayed, control tubes were set up containing the same components but with the addition of 0.2mM β-APN to inhibit lysyl oxidase activity. Where possible all reactions were carried out in duplicate.

2.2 Purification of collagen I monomers

2.2.1 Materials

Male Sprague-Dawley rats were supplied by Banton and Kingman, Universal Ltd, Field Station, Grimston, Hull. PMSF, NEM and β-APN (fumarate salt) were obtained from Sigma (Poole, Dorset). DEAE-Sephacel was supplied by Pharmacia/LKB (Milton Keynes, Bucks). Unless otherwise stated, all other reagents (analytical grade) were supplied by BDH (Poole, Dorset).

2.2.2 Procedure

The method was a modification of that of Macbeath *et al.* (1993) based on that of Payne *et al.* (1986). All procedures were carried out at 4°C and in the early stages of the purification, buffers contained a cocktail of protease inhibitors (25mM EDTA, 1mM PMSF, 10mM NEM and 1µg/ml pepstatin), together with 50µg/ml β-APN and 0.01% (w/v) sodium azide.

Twelve 21 day old male Sprague-Dawley rats were fed on a diet of rat chow and water. The water contained 0.17% (w/v) β-aminopropionitrile (β-APN), a lathyrogen which irreversibly inhibits lysyl oxidase activity and thus the crosslinking of collagen. This in turn increases the extractability of native collagen monomers. After 3 weeks on this diet the rats were sacrificed, shaved and the skins were removed. The skins (700g) were then cut into small pieces, passed through a pre-chilled mincer and homogenised at 2ml/g wet weight tissue in 1.08mM Na_2HPO_4, 0.22mM KH_2PO_4, pH

7.4 in a Waring blender at high speed for 30 seconds. The mixture was centrifuged for 30 minutes at 10,000*g* and the pellet was re-homogenised and centrifuged before extraction overnight (16 hours) in 2ml/g 150mM NaCl, 5.4mM Na_2HPO_4, 1.1mM KH_2PO_4, pH 7.4.

After centrifugation, the supernatant was passed through 3 layers of cheesecloth to remove fatty material and further clarified by centrifuging at 18,000*g* for 2 hours. The supernatant was retained and solid NaCl was slowly added with stirring to give a final concentration of 2M. The mixture was left overnight to allow a precipitate to form. Collagen III precipitates out of neutral solutions at 2M NaCl (Epstein, 1974). The precipitate was pelleted by centrifugation at 10,000*g* for 30mins. To isolate collagen I from other contaminants, the supernatant was subjected to a further salt precipitation with the addition of solid NaCl up to a final concentration of 3.5M. The solution was again left overnight to allow a precipitate to form and centrifuged as before, after which the supernatant was discarded. The pellet was gently homogenised in a glass/Teflon homogeniser with a small volume of phosphate buffer (62mM Na_2HPO_4, 14.5mM KH_2PO_4, pH 7.4) and then redissolved in a larger volume of this buffer overnight. Insoluble material was removed by centrifugation at 65,000*g* for 4 hours at 4°C.

A second 3.5M NaCl salt precipitation was carried out as described previously and the precipitate was redissolved in and dialysed against 2 changes of DEAE start buffer (0.2M NaCl, 50mM Tris-HCl, pH 7.5). The extract was clarified by centrifuging at 10,000*g* for 30 minutes before loading onto a DEAE Sephacel anion exchange column (2.6 x 20cm) at a flow rate of 30ml/ hour. Under these conditions acidic contaminants such as proteoglycans are retained whilst collagen passes straight through the column. The absorbance of the unbound fractions were monitored using a two channel 276nm UV detector (Uvicord) and chart recorder. After sample loading was complete the column was washed through with DEAE start buffer until no further UV absorbing material was eluted.

The collagen solution was dialysed exhaustively against 0.02M Na_2HPO_4 until a white precipitate formed, followed by centrifugation at 10,000*g* for 30 minutes. The

pellet was redissolved in 0.5M acetic acid overnight and dialysed against the same solution. A further salt precipitation was then carried out to remove high molecular weight aggregates (Chandrakasen *et al.*, 1976), with the drop-wise addition of 20% (w/v) NaCl to a final concentration of 3.5% (w/v). After 15 minutes the resulting precipitate was centrifuged as above and the supernatant dialysed against two changes of 0.5M acetic acid followed by 4 changes of 5mM acetic acid. Finally the solution was centrifuged at 300,000*g* for 1 hour to produce a clear solution of collagen I which was stored in 0.5ml aliquots at -70°C and re-centrifuged just prior to use.

The purity of the collagen solution and its suitability for fibrillogenesis experiments was assessed by discontinuous SDS-PAGE in reducing and non-reducing conditions on 6% (w/v) acrylamide separating gels with 4% (w/v) stacking gels (section 2.3).

2.3 SDS-PAGE

2.3.1 Procedure

The method first described by Laemmli (1970) was used for discontinuous SDS-PAGE of proteins. For separation of proteins with molecular weights in the range 100kDa to 15kDa, 12% (w/v) acrylamide/0.32% (w/v) bisacrylamide separating gels were prepared and, for those in the range 200kDa to 60kDa, 6% (w/v) acrylamide/0.32% (w/v) bisacrylamide separating gels were used. For detailed procedures for preparation of gels, samples and electrophoresis see Hames (1990). For SDS-PAGE under reducing conditions samples contained 3% (v/v) β-mercaptoethanol whilst under non-reducing conditions samples did not contain β-mercaptoethanol. Electrophoresis was carried out in a water cooled LKB vertical gel electrophoresis apparatus at a constant current of 45mA per gel, with an LKB 2002 power supply. The gels were run for 3 hours or until the tracking dye front was 1cm from the bottom of the gel. Gels were then fixed and stained or taken for western blotting. When high resolution was not required, mini-gels were run on an LKB midget electrophoresis unit with water cooling, with run times and staining/destaining times correspondingly decreased.

2.3.2 Gel Staining Methods

2.3.2.1 Coomassie blue staining

To visualise protein bands, gels were fixed and stained with 0.25% (w/v) Coomassie Brilliant Blue R-250, 45% (v/v) methanol, 10% (v/v) acetic acid for 15 to 20 hours shaking slowly on a Denley reciprocal shaker. The gel was then destained with several changes of 7.5% (v/v) acetic acid, 5% (v/v) ethanol over 12 hours. The destaining process could be speeded up by heating the destaining solution to 50°C.

2.3.2.2 Silver Staining

Coomassie blue staining is a relatively insensitive staining method, the lower limit of detection being 0.2 - 0.5 µg protein (Hames, 1990). For detection of smaller amounts of protein, silver staining was used. The method was adapted from Morrissey (1981). Gels were fixed in 45% (v/v) methanol, 10% (v/v) acetic acid overnight, washed in distilled water for 1 hour and then incubated in distilled water containing 5µg/ml dithiothreitol for 2 hours to reduce proteins in the gel. This solution was removed and the gel was incubated for a further 2 hours in 0.1% (w/v) silver nitrate. After washing with distilled water, the gel was developed in 200ml of 3% (w/v) sodium carbonate containing 100µl of 40% formaldehyde until the desired level of staining was reached. The reaction was then stopped with the addition of 10ml 2.3M citric acid. After approximately 30 minutes the gel was placed in 2% (v/v) glycerol prior to photography or drying down.

2.3.2.3 Alcian Blue staining

Alcian blue 8-GX is a cationic dye which stains polyionic groups such as the negatively charged glycosaminoglycan (GAG) chains of proteoglycans (Scott, 1985). Macbeath *et al.* (1993) showed that TRAMP can also be stained with Alcian Blue. This is probably due to the interaction of the dye with the sulphate groups within

TRAMP since in 50mM $MgCl_2$, Alcian Blue is relatively specific for sulphated biopolymers (Scott, 1973).

For Alcian Blue staining after SDS-PAGE the method of Wall and Gyi (1988) was used. Gels were fixed for 1 hour in 50% (v/v) methanol, 7% (v/v) acetic acid and washed in distilled water for 1 hour. This cycle was repeated once and the gel was then stained with 0.2% (w/v) Alcian Blue 8-GX, 3% (v/v) acetic acid, 0.05M $MgCl_2$ overnight. Destaining was carried out in several changes of 3% (v/v) acetic acid, 0.05M $MgCl_2$. Addition of $MgCl_2$ prevents the high levels of background staining usually observed for Alcian Blue.

2.4 Western blotting

Electrophoretic blotting and immunological detection of proteins was performed using the method first described by Towbin *et al.* (1979).

2.4.1 Transfer of proteins to nitrocellulose

2.4.1.2 Electrophoretic transfer

Immediately following SDS-PAGE, the gel was removed from the glass plates, marked uniquely by removing the bottom right hand corner and placed in blotting buffer (20mM Na_2HPO_4, 20% (v/v) methanol, pH 9.4) for 1-2 minutes. A gel-nitrocellulose 'sandwich' was then assembled as follows, making sure there were no air bubbles. The open clamp assembly of a Biorad TRANS-BLOT cell was placed in a basin of blotting buffer with a scotch-brite pad, followed by a piece of 3MM Whatman filter paper cut to the size of the gel, then the gel itself and then a piece of pre-soaked 0.2µm nitrocellulose membrane (Sartorius) of the same size with the bottom right hand corner removed and finally a second piece of 3MM Whatman filter paper and a second scotch-brite pad. The clamp assembly was then closed and placed into the transfer tank containing blotting buffer with the gel side of the sandwich nearest the negative electrode. The transfer of proteins from the gel to the

nitrocellulose was completed by running the apparatus for 16 hours at 250mA with maximum voltage set to 20V.

2.4.1.2 Dot blotting

Where speed rather than sensitivity was the priority, the dot blotting procedure (Hawkes, 1986) described below was carried out. The base of the BIO-DOT microfiltration unit (Biorad) was filled with dH_2O and the tap was opened briefly to ensure the tubing was also filled. The centre of the blotter was placed into the base and the silicon rubber gasket was then placed on top of this assembly. The nitrocellulose membrane (12.5 x 9.5cm) which had been presoaked in dH_2O was then placed on top of the gasket before replacing the top of the apparatus and tightening the screws. One hundred µl of the samples to be tested were added to the appropriate wells and empty wells were filled with an equal volume of dH_2O. The tap was then opened and the apparatus was slowly drained. A vacuum was applied to the dot blotter for 1 minute before closing the tap and slowly releasing the vacuum. Finally, the nitrocellulose was removed and proteins were detected as described below.

2.4.2 Detection of proteins by enhanced chemiluminescence (ECL)

To check that proteins had been transferred to the nitrocellulose, the membrane was soaked in 0.4% (w/v) Ponceau S in 3% (w/v) TCA for 1 minute and then washed with several changes of dH_2O to visualise protein bands. The membrane was blocked with 5% (w/v) dried milk in TBST (50mM Tris-HCl, 0.15M NaCl, 0.05% (v/v) Tween-20, pH 7.9) for 1 hour with shaking and washed in TBST for 1 x 15 minutes followed by 2 x 5 minutes. The primary antibody diluted with TBST was incubated with the membrane for 90 minutes with shaking. The washing step was repeated before addition of a 1:5000 dilution of horseradish peroxidase conjugated anti-rabbit IgG second antibody (SAPU) for a further hour. The washing step was again repeated before antigen detection with Amersham ECL reagents mixed in a 1:1 ratio and incubated with the membrane for exactly 1 minute. The membrane was then placed

onto a piece of Whatman 3MM filter paper, covered with cling film, placed in a Kodak X-Omatic cassette and immediately exposed to Kodak X-OMAT AR scientific imaging film for up to 15 minutes, depending on amount of antigen being detected and dilution of the primary antibody. All procedures were carried out at room temperature.

2.4.3 Stripping nitrocellulose membranes

When necessary blots could be stripped and reprobed using a modification of the method of Kaufman *et al.* (1987). The membrane was submerged in stripping buffer (100mM β-mercaptoethanol, 2% (w/v) SDS, 62.5mM Tris-HCl, pH 6.7) and incubated at 50°C for 30 minutes. The membrane was then washed in TBST for 1 x 15 minutes, 1 x 10 minutes and 1 x 5 minutes before repeating western blotting procedure with the new primary antibody.

2.5 Densitometry

To quantify the results of SDS-PAGE separations, stained gels were scanned using a Joyce-Loebl Chromoscan 3 coupled to a DCS microcomputer for digital analysis. The chromoscan was operated in absorbance mode with an aperture of 0.3 x 5mm. The light source was a 100 W tungsten halogen lamp and scans were carried out with a red (626nm) filter for Coomassie blue stained gels and a green (530nm) filter for Alcian blue stained gels. Before calculating the integrated peak areas, a background subtraction was made.

2.6 Protein assays

2.6.1 Bicinchoninic acid (BCA) protein assay

Protein concentrations were measured using the BCA protein assay (Pierce). This method, first described by Smith *et al.* (1985), combines the ability of proteins to reduce Cu^{2+} to Cu^{1+} in alkaline conditions, with BCA, a detection reagent for Cu^{1+}.

2.6.1.1 Procedure

The microtiter plate protocol (Redinbaugh and Turley, 1986) was used with standards in the range 0 to 250µg/ml BSA. All samples were run in duplicate. Working reagent was prepared by mixing 50 parts solution A containing the BCA detection reagent with 1 part solution B (4% copper sulphate). Ten µl of each standard, blank or sample was placed in the appropriate microtiter plate well and 200µl of the working reagent was added using a digital multi-channel pipette (Labsystems). The plate was then covered and incubated at 60°C for 30 minutes. After cooling to room temperature the absorbance at 490nm was measured in a Dynatech MR7000 microplate reader. Protein concentrations of unknown samples were calculated automatically from the standard curve of varying BSA concentrations using Dynatech Data Reduction software.

2.6.2 Measurements of collagen concentration

2.6.2.1 Sircol collagen assay

Collagen concentrations were measured using the Sircol collagen assay (Biocolor). This is a quantitative dye binding assay based on Sirius red. The sulphonic acid side chain groups on Sirius red react with the side chain groups of the basic amino acids in collagen. It is believed that the specific affinity of the dye for collagen is due to the elongated dye molecules becoming aligned with the triple helical structure of collagen (Junqueira *et al.*, 1979).

2.6.2.1.1 Procedure

Standards were prepared in duplicate from a 1mg/ml stock solution of collagen I in 0.5M acetic acid (Coletica). Aliquots of 0, 12.5, 25 and 50µl of this stock were added to eppendorf tubes as was 50µl of each of the unknown samples. Acetic acid (0.5M) was added to give a final volume of 100µl. After addition of 1ml of Sircol dye reagent the tubes were mixed on a blood tube rotator for 30 minutes at room

temperature. The tubes were then centrifuged at 10,000*g* for 5 minutes and the supernatant containing unbound dye was removed. To further remove excess dye solution the tubes were inverted and wiped with an absorbant paper tissue taking care not to disturb the pellet. The pellet was then redissolved in 1 ml of 0.5M sodium hydroxide and the absorbance at 540nm for each sample was measured in a Pharmacia Biotech Ultraspec 2000 UV/VIS spectrophotometer using water as a blank. The collagen concentration for the unknown samples was calculated from the standard curve.

2.6.2.2 Hydroxyproline assay

The method of Woessner *et. al.* (1961) was used to measure the hydroxyproline content of samples and thus the amount of collagen present. 100µl of samples to be tested were placed in 2ml Multi-max Seal tubes (Sorenson Bioscience) and 1.2ml 6M HCl was added. The tubes were sealed and hydrolysed overnight at 107°C. Samples were transferred to glass tubes and dessicated to dryness for 24-48 hours under vacuum in a dessicator containing NaOH pellets and silica gel. Samples were then resuspended in 1ml of dH_2O and 100µl aliquots were removed and made up to 500µl. Hydroxyproline standards containing 0, 2.5, 5, 10, 20, 30, 40, 50 nmoles/ml were set up. A total of 500µl of standards and samples to be assayed were placed in fresh test tubes and 0.5ml of 2-methoxyethanol was added, followed by 0.2ml chloramine T solution (21mM citric acid, 335mM sodium acetate, 102mM trisodium acetate, 38.5% (v/v) 2-methoxyethanol, 2% (w/v) chloramine T, pH 6.0). After mixing, each tube was incubated at room temperature for 5 minutes. A volume of 0.3ml of Ehrlich's reagent (87.5% (v/v) 2-methoxyethanol, 12.5% (w/v) p-dimethylaminobenzaldehyde, 12.5% (v/v) concentrated HCl) was then added followed by incubation at 70°C for 15 minutes. Finally the tubes were cooled to room temperature and the absorbance at 553nm was measured in a Pharmacia Biotech Ultraspec 2000 UV/VIS spectrophotometer. The amount of hydroxyproline in each sample was calculated from the standard curve and the collagen content calculated assuming collagen I contains 10% hydroxyproline (Berg, 1982).

2.7 Affinity purification of TRAMP antiserum using TRAMP immobilised on nitrocellulose

The above method is useful for affinity purification of antibodies when purified antigen is not available since the protein of interest can be separated from contaminants by SDS-PAGE prior to blotting onto nitrocellulose (Smith and Fisher, 1984).

One ml of partially purified TRAMP (~1mg) was loaded onto a 12% acrylamide gel, with a single well spanning the width of the gel, and was subjected to electrophoresis (section 2.3). The proteins were then transferred to nitrocellulose as described previously (2.4.1) and visualised with 0.4% (w/v) Ponceau S in 3% TCA (w/v). The band corresponding to TRAMP was excised and the bottom right hand corner removed to allow identification of the antigen face. The affinity strip was blocked with TBS (50mM Tris-HCl, 0.15M NaCl, pH 7.4) containing 5% (w/v) dried milk, 1% (v/v) Tween-20 for 90 minutes with shaking. After washing the strip in TBS containing 1% (v/v) Tween-20 for 1 x 15 minutes and 2 x 5 minutes, the strip was placed on parafilm (antigen side up) in a humidity chamber (see figure 2.1).

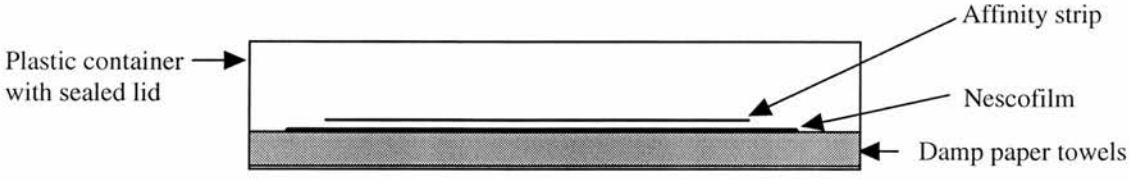


Figure 2.1. Schematic diagram showing humidity chamber layout for affinity purification of TRAMP antiserum.

As much antiserum as could be held on by surface tension (~250µl) was then placed on the strip and this was incubated at room temperature for 2 hours with gentle shaking. Excess serum was removed and the strip was washed with 250µl aliquots of TBST for 1 x 15 minutes and 2 x 5 minutes before eluting bound antibody from the strip by incubating with 250µl of 0.2M glycine-HCl, pH 2.8 for 20 minutes with shaking. This buffer was removed and immediately neutralised with an equal volume

of 0.1M Tris-HCl, pH 8.5. The elution and neutralisation steps were repeated twice more to remove as much affinity purified antibody as possible. After pooling the eluants, 0.1% (w/v) sodium azide was added and the affinity purified antibodies were stored at 4°C. The affinity strip was washed in TBST and stored for future use at 4°C, submerged in TBS containing 0.1% (w/v) sodium azide.

Chapter 3

Purification of TRAMP

3.1 Introduction

As discussed previously, TRAMP was first identified as a contaminant in lysyl oxidase preparations (section 1.6; Cronshaw *et al.*, 1993). The apparent close association of these proteins has hindered attempts to obtain high purity samples of TRAMP. Both Macbeath *et al.* (1993) and Cronshaw *et al.* (1993) devised methods involving urea extraction followed by DEAE anion exchange chromatography and an additional, unusual chromatography step based on the selective interaction of TRAMP and lysyl oxidase with Sephacryl S-200/S-400 media (Shackleton and Hulmes, 1990a). However, both these methods required further purification steps such as Mono Q FPLC and reverse phase HPLC to produce TRAMP of high enough purity for further analyses. This decreased yields, increased the time scale of purification and in the case of reverse phase subjected the protein to harsh denaturing conditions.

The aims of the present study were to produce highly purified, active TRAMP for fibrillogenesis and enzymatic studies, to improve yields and to decrease the time scale of the purification procedure.

3.2 Detection methods for lysyl oxidase and TRAMP

Generally, the purification process was monitored spectrophotometrically by measuring the absorbance at 280nm and also by SDS-PAGE followed by Coomassie blue or silver staining (section 2.3). Total protein concentrations were measured using the BCA microtiter plate assay (section 2.6). For specific detection of TRAMP, SDS-PAGE was followed by western blotting (section 2.4) using a 1:5000 dilution of polyclonal anti-serum to TRAMP (Forbes *et al.*, 1994). The presence of lysyl oxidase was monitored using the ultrafiltration assay described in section 2.1. In later purification attempts, an antibody to lysyl oxidase was made available (by Dr. M. Ouzzine) and thus detection was also carried out by western blot analysis.

3.3 Methods for buffer exchange and concentration

Throughout the purification a number of methods were used for desalting/buffer exchange of protein samples. The method used depended on the volume and concentration of the sample. For volumes above 1.5ml dialysis was used, whilst gel filtration on prepacked Sephadex G-25 HiTrap desalting columns (Pharmacia) was used for volumes between 200µl and 1.5ml. For sample volumes below 200µl, gel filtration was performed using Biogel P-6DG (Biorad) as follows. Briefly, ~1.5ml of preswollen Biogel was placed in an Eppendorf tube with a small hole punched in the base which was plugged with glass wool. After pre-equilibrating with the appropriate buffer, excess liquid was removed from the column by placing the tube in a glass centrifuge tube and centrifuging for 1 minute at 1000rpm in a MSE bench centrifuge. The sample was placed on top of the gel and left to stand for 1 minute before centrifuging for a further minute at 1000rpm to collect the protein sample. This method (courtesy of Dr. D. Apps) was particularly useful since sample dilution was minimal.

3.4 Purification methods

Unless otherwise stated all procedures were carried out at 4°C. Also, in initial stages of the purification, all buffers contained 1mM PMSF to inhibit serine protease activity.

3.4.1 Urea extraction

Urea extraction was carried out as previously described by Macbeath *et al.* (1993). The skins from 6 to 10 stillborn pigs were diced, fed through a pre-chilled mincer and homogenised in PBS (0.09M Na_2HPO_4, 0.01M NaH_2PO_4, 0.15M NaCl, pH 7.8) at 2ml/g in a Waring Blender for 3 x 20 seconds at high speed. The extract was then centrifuged at 10,000*g* for 20 minutes and the supernatant was discarded. This washing step was then repeated once with PBS and 3 times with PB (9mM Na_2HPO_4, 1mM NaH_2PO_4, pH 7.8) at 2ml/g with centrifugation as above. The pellet was then

extracted overnight in 6M PBU (9mM Na_2HPO_4, 1mM NaH_2PO_4, 6M urea, pH 7.8) at 1ml/g of original tissue, again centrifuged as above and the supernatant was retained. This extraction procedure was repeated twice (for 8 hours and overnight respectively), the supernatants were then pooled and filtered through Whatman No. 113V filter paper and further clarified by centrifugation at 12,000*g* for 1 hour.

3.4.2 DEAE-Sephacel anion exchange chromatography

DEAE anion exchange chromatography is often used in the initial stages of lysyl oxidase purification and thus has been incorporated into TRAMP purifications (Macbeath *et al.*, 1993; Cronshaw *et al.*, 1993).

In initial experiments the urea extract was loaded onto the DEAE-Sephacel column (Pharmacia; 2.6cm x 20cm), pre-equilibrated with 6M PBU, at a flow rate of 35ml/hour. The column was then washed with 1 column volume of 6M PBU followed by 2 column volumes of 10mM Bis-Tris containing 6M urea, pH 6.0 (flow rate 80ml/hour). Proteins were eluted from the column either by 1 step elution with 10mM Bis-Tris, 6M urea, pH 6.0 containing 0.5M NaCl as described by Macbeath *et al.* (1993) or with a salt gradient from 0 to 0.5 M NaCl in 10mM Bis-Tris, 6M urea, pH 6.0 at a flow rate of 60ml/hour. The stepwise elution was found (by SDS-PAGE and lysyl oxidase assay) to be unsuitable for removal of both lysyl oxidase and other contaminating proteins from TRAMP (data not shown). In contrast, during the gradient elution the majority of contaminating proteins were eluted from the column at salt concentrations of between 0 and 0.25M, whilst TRAMP was eluted towards the end of the gradient (Figure 3.1). However, it should also be noted that although some lysyl oxidase eluted towards the beginning of the gradient, a significant amount co-eluted with TRAMP (Figure 3.1a). Changing the pH of the Bis-Tris buffer to 7.0 had little affect on the elution profile or binding of lysyl oxidase.

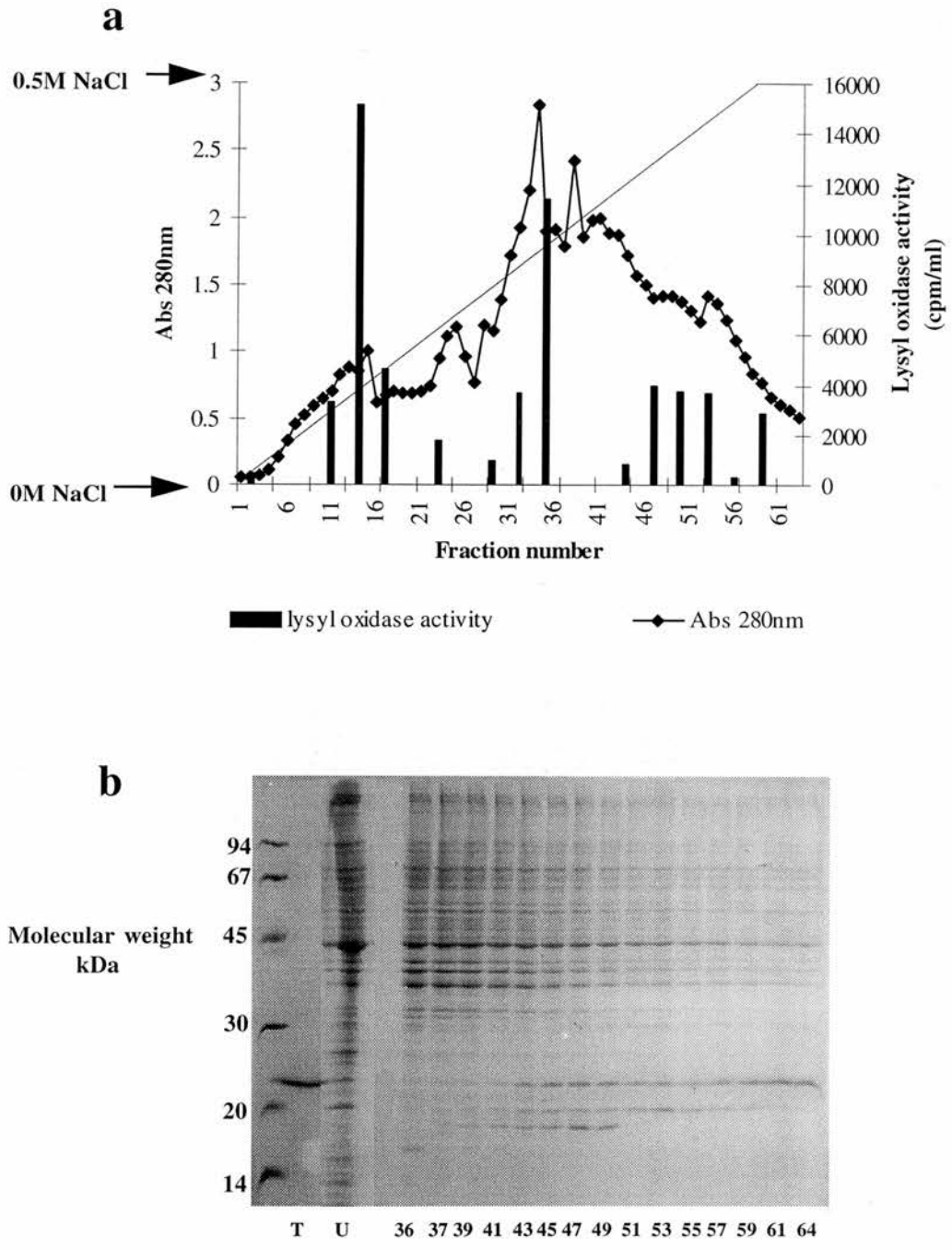


Figure 3.1. DEAE-Sephacel anion exchange chromatography of the urea extract. (a) Proteins were eluted from the column with a gradient of 0 to 0.5M NaCl in 10mM Bis-Tris, 6M urea, pH 6.0. Values for lysyl oxidase activity are expressed after subtraction of blanks containing 0.2mM β–APN (b) SDS-PAGE analysis of urea extract (U) and fractions (36 to 64) from DEAE-Sephacel column. T represents TRAMP standard.

3.4.3 Selective interaction with Sephacryl S-400 media

Following a stepwise elution from the DEAE-Sephacel column, fractions containing TRAMP were pooled, concentrated (in an Amicon stirred cell concentrator using a YM10 membrane) and dialysed against 3 changes of PB (9mM Na_2HPO_4, 1mM NaH_2PO_4, pH 7.8). Aliquots of this sample (1.5ml; 10mg) were loaded onto Sephacryl S-400 test columns (Pharmacia; 1.3 x 20cm), washed with PB and then eluted with either 6M PBU only or 1.5M PBU followed by 6M PBU (Cronshaw *et al.*, 1993). Elution with 6M PBU only, resulted in elution of both lysyl oxidase and TRAMP in a single fraction (data not shown). A small amount of TRAMP was detectable by SDS-PAGE and silver staining after elution with 1.5M urea. However, the majority of TRAMP remained bound to the column until addition of 6M urea led to the co-elution of TRAMP and lysyl oxidase (Figure 3.2).

3.4.4 Affinity purification on amino hexyl Sepharose

Amino hexyl Sepharose chromatography has been used for affinity purification of a number of mammalian copper amine oxidases, including bovine serum amine oxidase (BSAO; Houen *et. al.*, 1993). A 1,6-diaminohexane spacer group covalently linked to Sepharose 4B acts as the ligand for binding of amine oxidases which can then be eluted, depending on the strength of binding, by increasing the ionic strength of the buffer or by addition of octylamine. Since lysyl oxidase is also a copper amine oxidase the same method was used in an attempt to separate it from TRAMP.

A sample volume of 25ml (~4.5mg protein) of the DEAE fraction after stepwise elution was dialysed against 4 changes of 10mM PB, pH 7.2 and loaded onto an amino hexyl (EAH) Sepharose 4B column (Pharmacia; 1.6 x 5cm) pre-equilibrated with the same buffer. After washing with PB to remove unbound material, proteins were eluted with a gradient of 10mM to 100mM PB, pH 7.2 followed by 100mM PB, pH 7.2 containing 10mM octylamine. The major peak of lysyl oxidase activity was found at the very end of the gradient prior to addition of octylamine (Figure 3.3a).

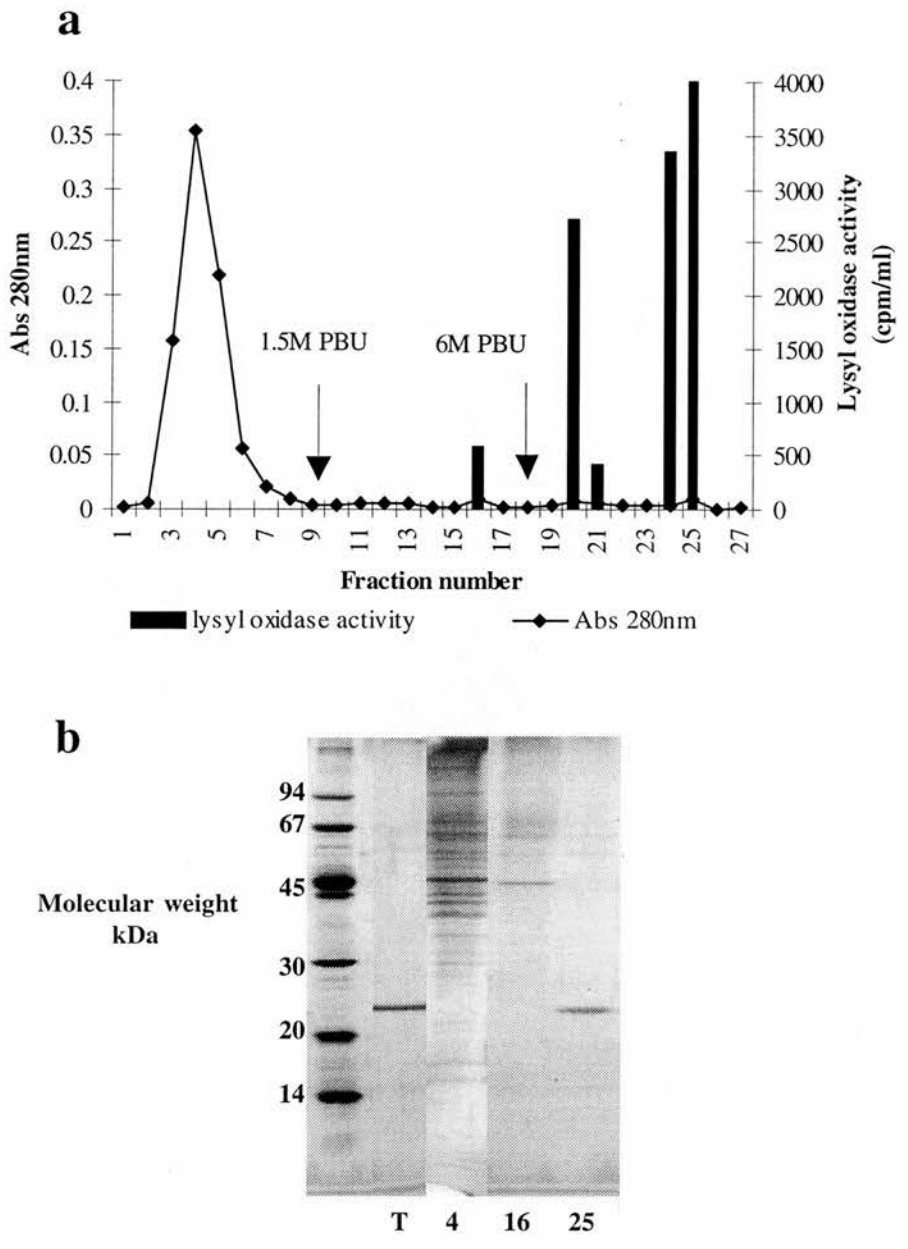


Figure 3.2. Sephacryl S-400 chromatography of pooled fractions from DEAE-Sephacel. (a) Elution profile from Sephacryl S-400 column. Addition of 1.5M and 6M PBU is denoted by arrows. Fractions 4 and 16 to 25 were assayed for lysyl oxidase activity and values obtained are expressed after subtraction of blanks containing 0.2mM β–APN. (b) Peak fractions (4, 16 and 25) were analysed by SDS-PAGE followed by silver staining. T represents TRAMP standard.

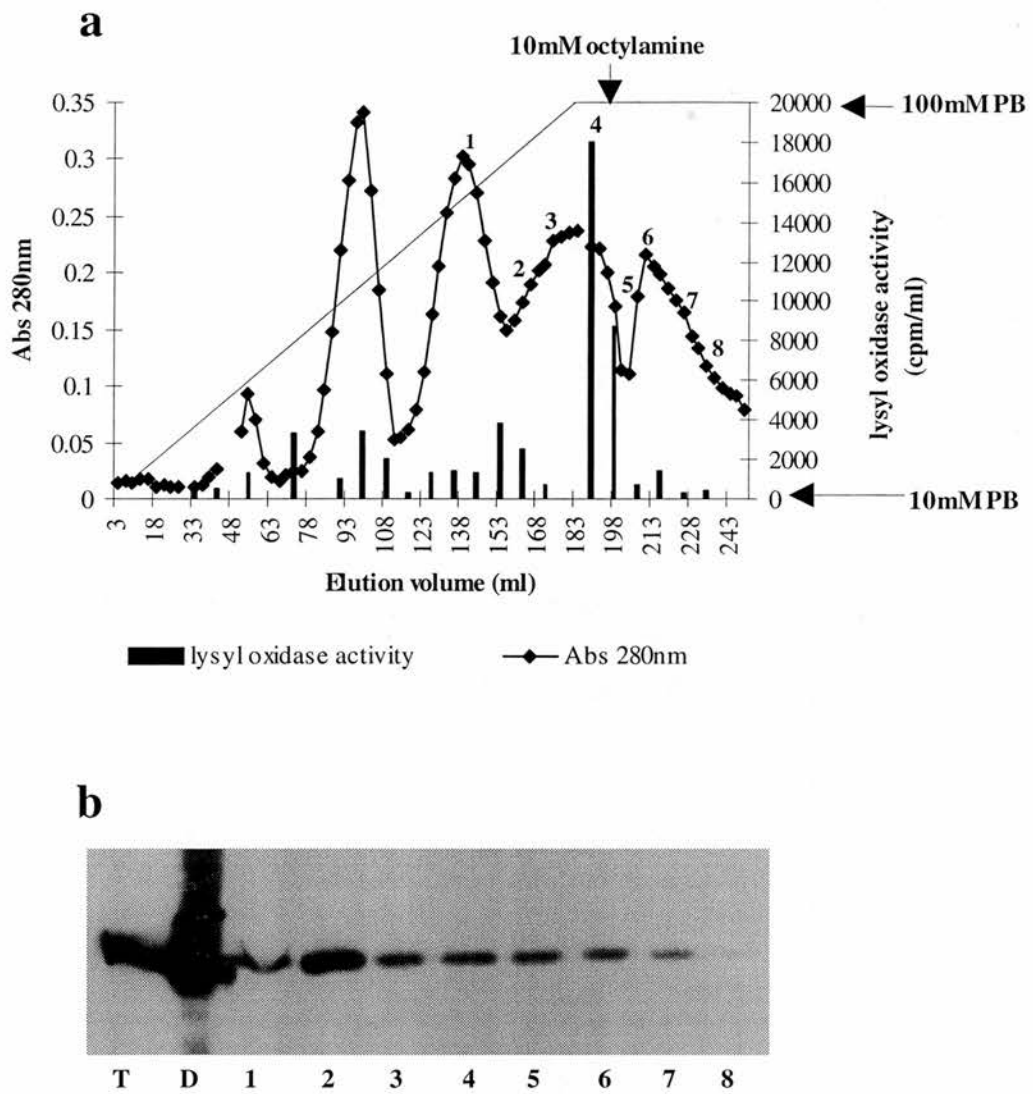


Figure 3.3. Amino hexyl Sepharose chromatography of the DEAE 0.5M NaCl eluate. (a) Elution profile is shown with a gradient from 10 to 100mM PB, pH 7.2 (diagonal line) followed by addition of 10mM octylamine (vertical arrow). Lysyl oxidase activities are expressed after subtraction of blanks containing 0.2mM β–APN. (b) Western blot analysis identified fractions containing TRAMP. T represents TRAMP standard, D the DEAE fraction prior to loading and lanes 1 to 8 correspond to fractions labelled 1 to 8 in (a).

Western blot analysis showed the presence of TRAMP in all fractions after ~70mM PB (Figure 3.3b). It should be noted that octylamine containing fractions were not dialysed to remove octylamine prior to use in the lysyl oxidase assay. This may have resulted in a false negative result for the absence of lysyl oxidase in fractions eluted after addition of octylamine.

3.4.5 Preparative isoelectric focusing

The Biorad Rotofor cell can be used for preparative isoelectric focusing in free solution and is based on separation of proteins by differences in their isoelectric points (pI). This method has previously been reported to separate proteins with pI differences of about 0.6 (Curnette *et al.*, 1989) and manufacturer's literature suggest that by narrowing the pH range of the ampholytes used, proteins with differences in pI values of as little as 0.1 could be resolved. It would thus appear to be a powerful technique for the separation of TRAMP and lysyl oxidase.

An aliquot of the DEAE step-wise eluate (~8mg protein) in 10mM Bis-Tris, 3M urea, pH 7.0 (58ml) was mixed with a Biolyte ampholytes mixture (Biorad; 5 parts Biolytes 3/10 and 1 part Biolytes 3/5) to give a final ampholyte concentration of 2%. The sample was focused in a Biorad Rotofor cell according to the manufacturer's instructions at 15 Watts constant power until a constant voltage was maintained (~ 4 hours). Fractions were collected by suction and were analysed by SDS-PAGE and lysyl oxidase assay.

Figure 3.4a shows the peak of lysyl oxidase activity in fraction 5 which corresponds to a pH of 4.61. This compared to pI values for lysyl oxidase obtained by analytical IEF of between 5.2 and 5.8 (Cronshaw, 1993; Macbeath, personal communication). Electrophoretic analysis showed TRAMP to be present in fractions 3, 4 and 5 corresponding to a pH range of 4.01 to 4.61 (Figure 3.4b). This is in agreement with the pI values calculated by analytical IEF for the 5 variants of TRAMP which are in the range 4.07 to 4.43 (Macbeath *et al.*, 1993). The similarity in the apparent pI values of lysyl oxidase and TRAMP precluded the use of preparative isoelectric focusing as a separation technique.

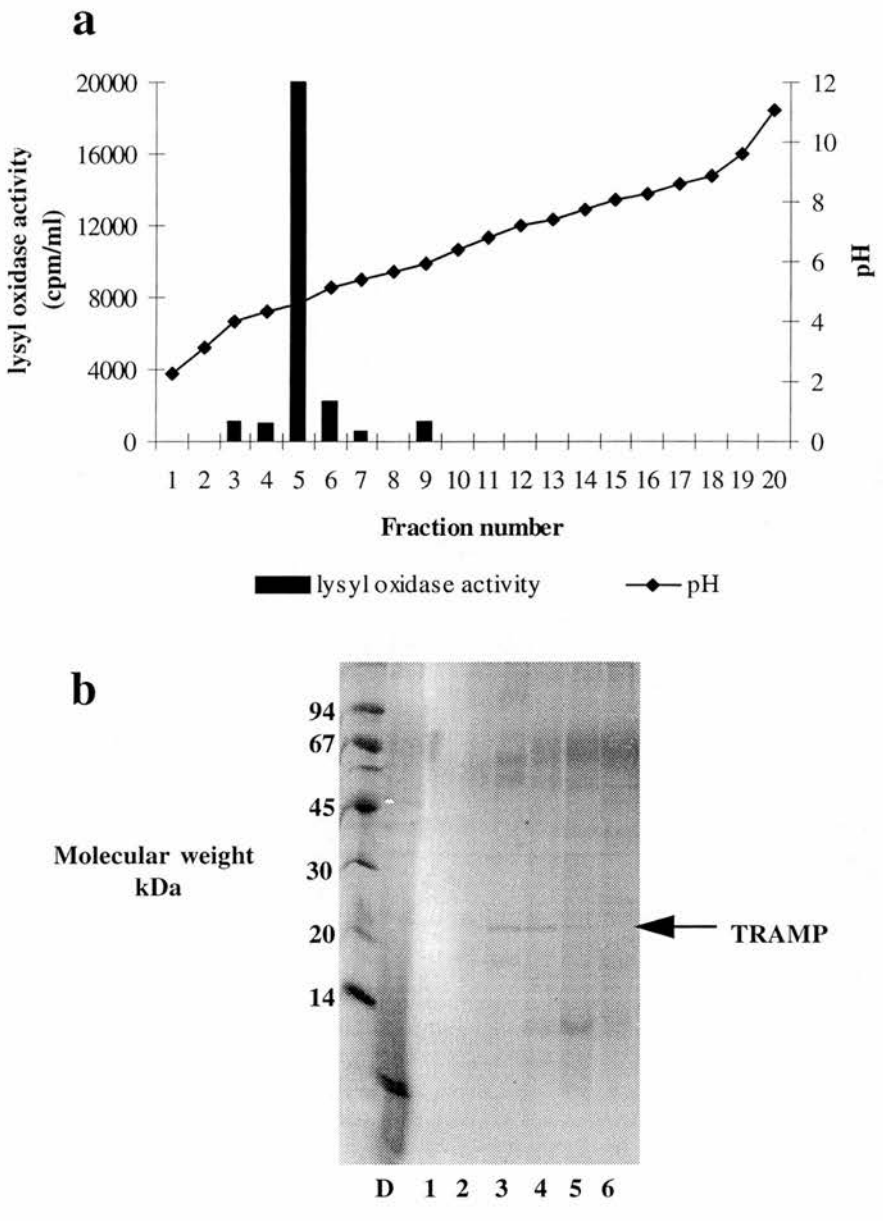


Figure 3.4. Preparative isoelectric focusing of DEAE fractions in Biorad Rotofor cell. (a) Graph showing pH gradient and lysyl oxidase activity (after subtraction of blanks containing 0.2mM β–APN) of fractions after IEF. Fractions 3 to 10 were assayed for lysyl oxidase activity. (b) SDS-PAGE and Coomassie staining of fractions 1 to 6 and the DEAE fraction prior to focusing (D).

3.4.6 Superdex-75 size exclusion chromatography

Superdex-75, comprised of highly crosslinked porous agarose beads onto which dextran is covalently bonded, separates proteins within the molecular mass range 3000 to 70,000 daltons.

Following gradient elution from the DEAE-Sephacel column, fractions containing TRAMP, as judged by SDS-PAGE and Coomassie staining, were pooled (~ 400ml) and concentrated to between 1 and 5ml using a YM10 membrane in an Amicon stirred cell concentrator, followed by further concentration with Centricon-10 microconcentrators centrifuged at 5,000*g*. The concentrated sample (maximum volume, 3.5ml) was then applied to a Pharmacia prepacked Hiload Superdex-75 size exclusion column (2.6cm x 60cm), pre-equilibrated with 6M PBU, pH 7.8 containing 0.5M NaCl at room temperature. Proteins were eluted from the column with this same buffer at a flow rate of 20 ml/hour and fractions were collected every 30 minutes. Fractions were analysed by SDS-PAGE and lysyl oxidase assay. Figure 3.5a shows the elution profile from the Superdex-75 column after loading 3.5ml of the concentrated protein (60mg). There were 3 major peaks of absorbance at 280nm. SDS-PAGE followed by Coomassie blue staining showed the first peak (V_e, 100ml) to contain high molecular weight material (Figure 3.5b, lane 1) whilst the third peak (V_e, 210ml) appeared to contain low molecular weight material which could not be visualised on a 12% acrylamide separating gel (data not shown). A single protein band of ~22kDa, corresponding to TRAMP, was observed in fractions from the second peak (V_e, 150ml; Figure 3.5b, lanes 2-4). However, lysyl oxidase co-eluted with TRAMP (V_e, 150ml), although the major peak of lysyl oxidase activity was eluted after 140ml (Figure 3.5a, peaks 2 and 3). Thus despite the use of a relatively low flow rate to improve resolution, the similarity in the molecular weights of TRAMP and lysyl oxidase precluded their separation under these conditions.

Partial separation of the two proteins was only obtained after decreasing both the sample volume and the total amount of protein loaded. Figure 3.6a shows the elution profile after loading a 1.5ml sample containing 25mg total protein. The peak of lysyl

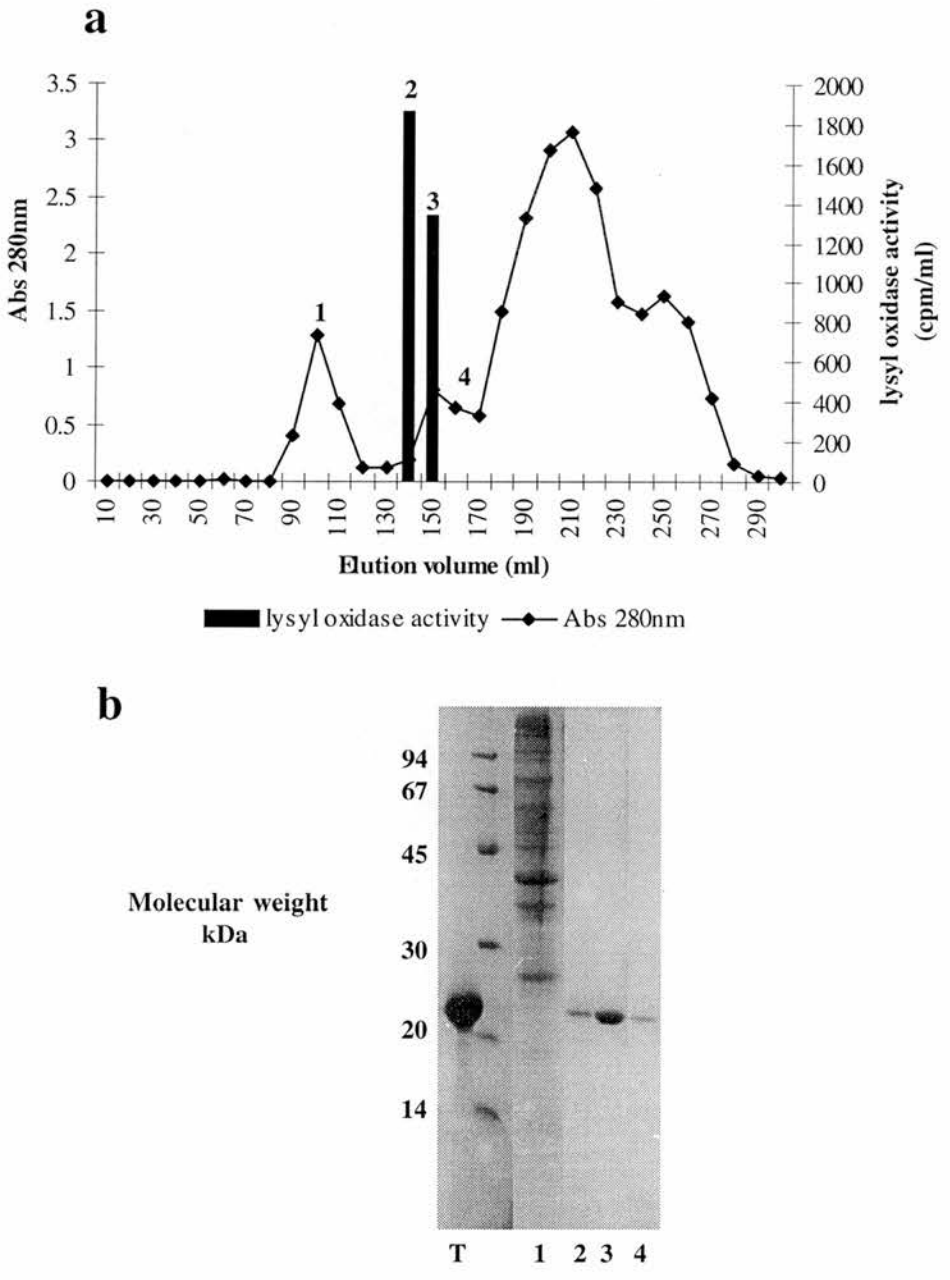


Figure 3.5. Superdex-75 size exclusion chromatography in the presence of urea. (a) Elution profile after loading 3.5ml (60mg) of pooled, concentrated DEAE fraction at a flow rate of 20ml/hour. Fractions with V_e between 120 and 180ml were assayed for lysyl oxidase activity (values are expressed after subtraction of blanks containing 0.2mM β–APN). (b) SDS-PAGE followed by Coomassie blue staining of peak fractions labelled 1 to 4 in (a). T represents TRAMP standard.

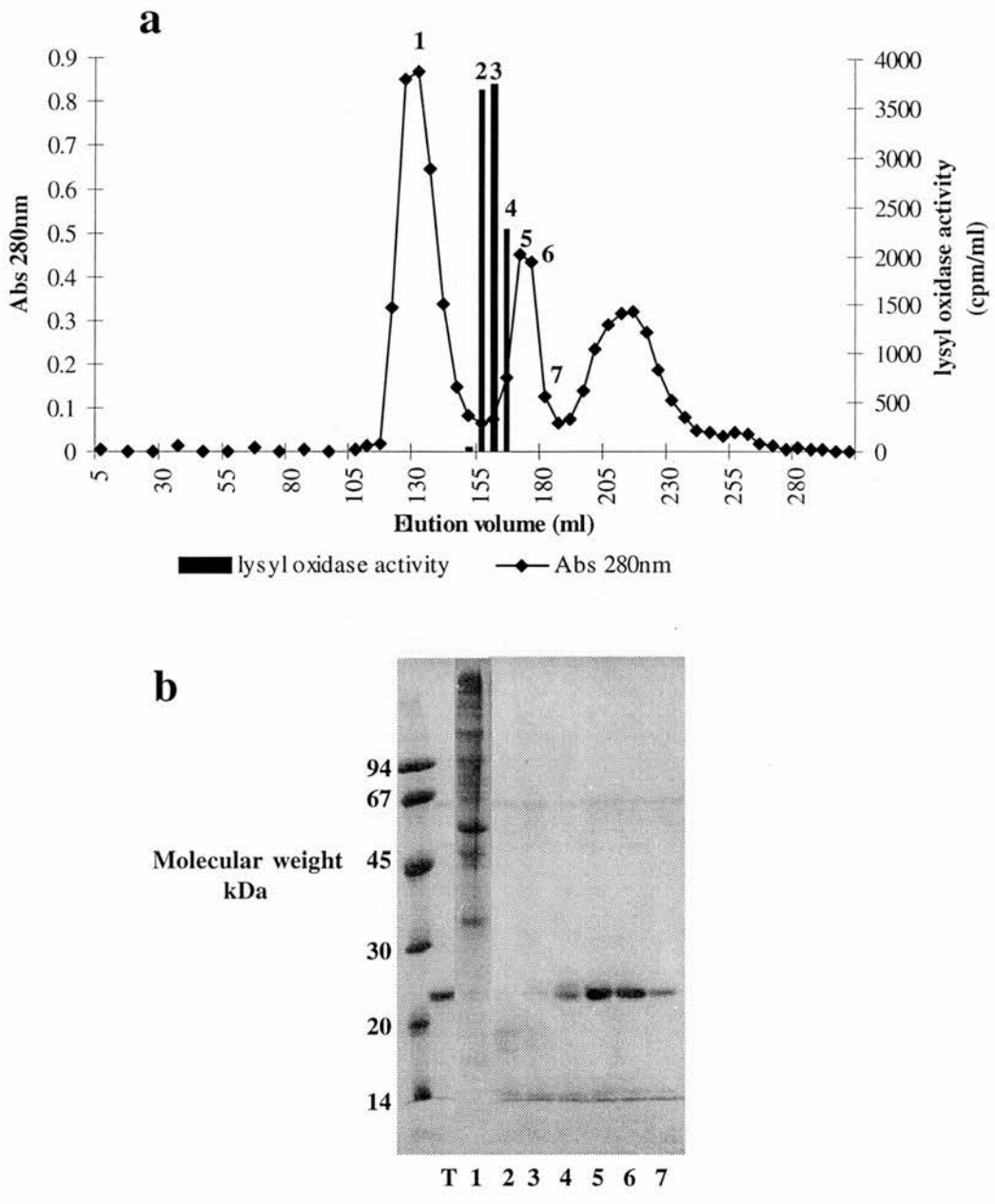


Figure 3.6. Superdex-75 size exclusion chromatography in the presence of urea. (a) Elution profile after loading 1.5ml (25mg) of pooled, concentrated DEAE fraction in 20mM sodium phosphate, 0.5M NaCl, 6M PBU, pH 7.8. Flow rate 20ml/hour. Fractions 2 to 7 were assayed for lysyl oxidase activity (b) SDS-PAGE followed by Coomassie blue staining of peak fractions labelled 1 to 7 in (a). T represents TRAMP standard.

Protein	Elution Volume (ml)	K_{av}	Molecular weight (Da)
Blue Dextran 2000	118.4	-	-
Albumin	127.4	0.045	63,300
Ovalbumin	136.5	0.091	42,700
Chymotrypsinogen A	172.9	0.273	19,500
Ribonuclease A	191.1	0.363	15,500
Lysyl oxidase	160	0.208	28,140*
TRAMP	172	0.268	21,517*

Table 3.1. Calibration of Superdex-75 size exclusion column. Calibration was carried out according to the manufacturers instructions using a Pharmacia LMW calibration kit. For calculation of K_{av} values, V_o and V_t were taken as 118.4ml and 318.6ml respectively. *The molecular weights of lysyl oxidase and TRAMP were calculated from a graph of K_{av} v log MW for protein standards.

oxidase activity eluted between 155 and 165ml with the majority of TRAMP eluting between 165 and 185ml. The Superdex-75 column was calibrated with a Pharmacia low molecular weight calibration kit (containing blue dextran, ribonuclease A, chymotrypsin A, ovalbumin and albumin) and gave molecular weight values for lysyl oxidase and TRAMP of 28 and 21.5kDa respectively (Table 3.1). Thus, in the presence of urea, both TRAMP and lysyl oxidase were eluted according to their molecular weights. Purification of TRAMP by this method resulted in yields of 10μg/g wet weight starting material (Table 3.2).

An alternative method of separating TRAMP and lysyl oxidase by gel filtration was attempted, based on the suggestion that lysyl oxidase forms aggregates in the absence of urea (Jordan *et al.,* 1977). Thus, running the Superdex-75 column in 10mM PB, pH 7.8 containing 0.5M NaCl but in the absence of urea should result in the elution of aggregated lysyl oxidase in the void volume whilst the elution volume of TRAMP should remain unchanged. However, Figure 3.7 shows this not to be the case. The elution volume of lysyl oxidase was between 230ml and 290ml whilst TRAMP eluted between 300 and 360ml. The delayed elution of both proteins, compared to that observed in the presence of urea, suggests weak binding to the column. The yields obtained by this method were 6μg TRAMP per gram wet weight of starting material (Table 3.3).

3.4.7 Hydrophobic interaction chromatography

Hydrophobic interaction chromatography is based on the interactions between hydrophobic regions within proteins and immobilised hydrophobic ligands under conditions of high salt. Thus in general, proteins are loaded onto the column in high salt and eluted with a gradient of decreasing salt concentration (Roe, 1989).

In an attempt to further improve yields of TRAMP, Superdex-75 fractions containing a mixture of TRAMP and lysyl oxidase were subjected to hydrophobic interaction chromatography using a Gilson HPLC system at room temperature. Fractions from the Superdex-75 column containing both TRAMP and lysyl oxidase were subjected

	Purification step	Volume (ml)	[Protein] µg/ml	Total protein (mg)	Lysyl Oxidase Present	Yield (µg TRAMP /g wet weight starting material
1	Urea Extraction	1190	625	744	✓	-
2	DEAE-Sephacel	1180	103	121	✓	-
3	Concentration	3	19333	59	-	-
4	Superdex-75 (1)	30	126	3.8	X	**10**
5	Superdex-75 (2)	25	128	3.2	✓	-
6	Concentration and desalting	2	908	1.8	✓	-
7	Octyl Sepharose HIC	10	120	1.2	X	**1.5**
4 & 7	-	-	-	-	X	**11.5***

Table 3.2. Purification table showing results of a typical TRAMP preparation with separation by DEAE-Sephacel anion exchange and Superdex-75 size exclusion chromatography in the presence of urea. Superdex-75 (1) represents fractions free from lysyl oxidase whilst Superdex-75 (2) represents fractions containing both TRAMP and lysyl oxidase and thus taken for further purification.

*Represents combined yield from Superdex-75 (1) and HIC steps.

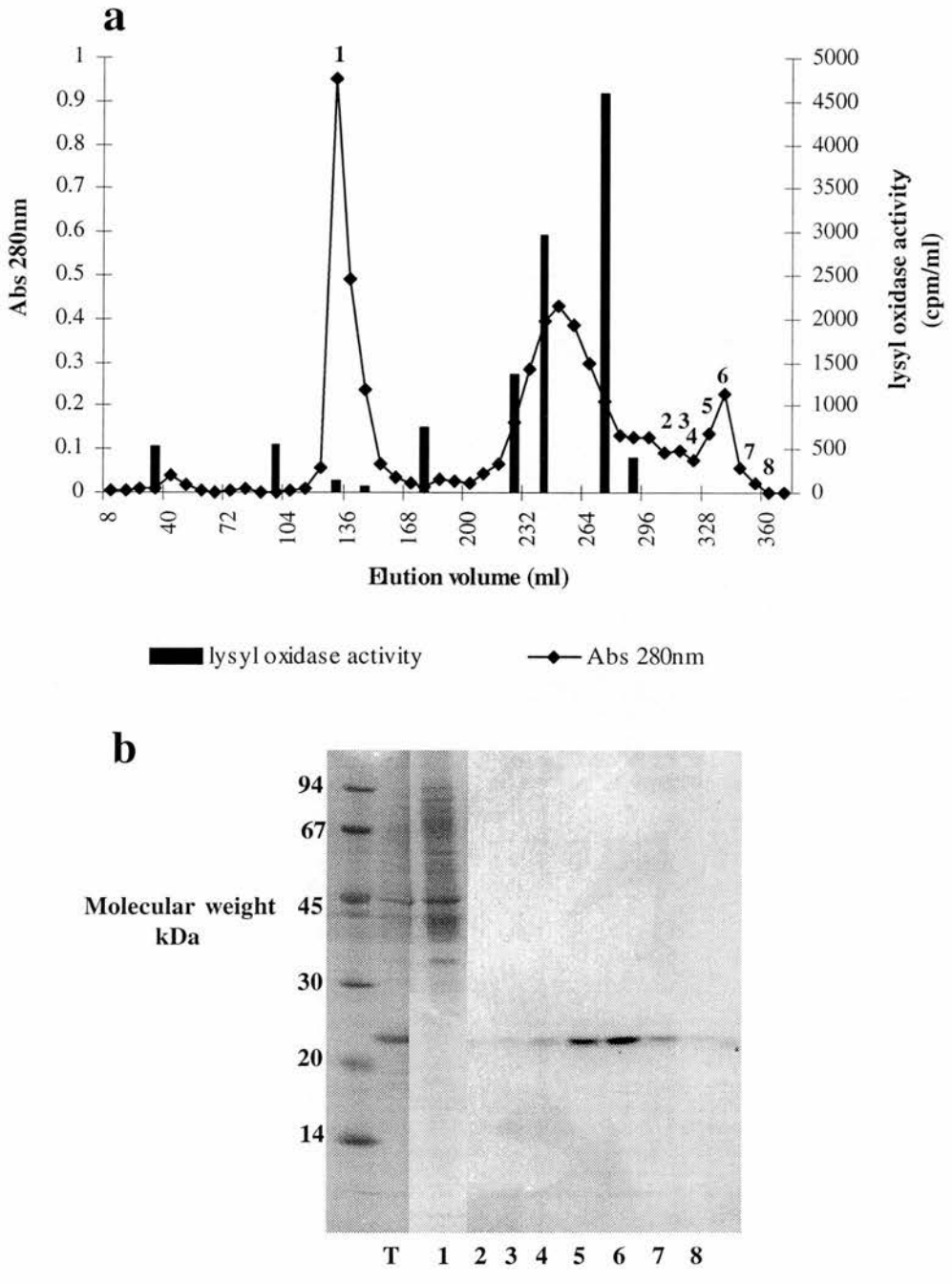


Figure 3.7. Superdex-75 size exclusion chromatography in the absence of urea. (a) Elution profile after loading 1.5ml (45mg) of pooled, concentrated DEAE fraction, dialysed into 20mM sodium phosphate, 0.5M NaCl, pH 7.8. Alternate fractions were assayed for lysyl oxidase activity and values were expressed after subtracting blanks containing 0.2mM β–APN. (b) SDS-PAGE followed by Coomassie blue staining of peak fractions 1-8 as labelled in (a). T represents TRAMP standard.

Purification Step	Volume (ml)	[Protein] μg/ml	Total Protein (mg)	Lysyl Oxidase Present	Yield (μg TRAMP/g wet weight starting material
Urea Extraction	245	652	160	✓	-
DEAE-Sephacel	375	89	61	✓	-
Concentration and Desalting	3.5	12800	33.3	✓	-
Superdex-75 (no urea)	60	77	4.6	X	**5.8**

Table 3.3. Purification table for separation of TRAMP by DEAE-Sephacel anion exchange and Superdex-75 size exclusion chromatography in the absence of urea.

to buffer exchange on a HiTrap desalting column pre-equilibrated with the appropriate starting buffer for hydrophobic interaction chromatography (section 3.3). Significant amounts of protein were lost during this procedure (Table 3.2) and a precipitate was observed in samples after buffer exchange, especially in the presence of 1M $(NH_4)_2SO_4$. The choice of media for hydrophobic interaction chromatography is not only dependent on the hydrophobicity of the amino acid sequence of a protein but also on knowledge of the number of hydrophobic amino acids which are surface exposed when the protein is folded into its tertiary structure (Roe, 1989). Thus, initial screening experiments were carried out using a HIC media test kit (Pharmacia) which is comprised of five 1ml prepacked columns containing the following HIC media: Phenyl Sepharose high performance, Phenyl Sepharose-6 fast flow (low substitution), Phenyl Sepharose 6 fast flow (high substitution), Butyl Sepharose 4 fast flow and Octyl Sepharose 4 fast flow. Using a Gilson HPLC system at room temperature, samples were loaded onto the column at 0.5ml/min in 50mM sodium phosphate, 1M ammonium sulphate, pH 7.0 and a gradient of decreasing salt was applied. Only the Octyl Sepharose 4 fast flow column was able to bind TRAMP. However, under these conditions the binding was so strong that the protein did not elute after removal of ammonium sulphate (data not shown). Additional attempts to elute TRAMP using 40% (v/v) ethylene glycol failed. Since the strength of binding of the protein is dependent on the initial salt concentration, the concentration of ammonium sulphate was lowered to 0.25M. Figure 3.8a shows the elution profile from such a separation attempt. A small absorbance peak of unbound material was observed in fraction 2 whilst the major peak was found at the very end of the gradient in fractions 27 to 40. This peak was found, by SDS-PAGE and Coomassie blue staining, to consist of 2 protein bands, one at 21kDa and another of around 16kDa (Figure 3.8b). Since the 16kDa band was also observed in the sample prior to loading it was believed to be a degradation product of TRAMP.

Detection of lysyl oxidase was carried out by western blotting with a 1:2500 dilution of the antiserum raised against recombinant human lysyl oxidase (a gift from Dr. M. Ouzzine). Prior to loading onto the column, 4 bands were observed on the blot at approximately 16, 21, 26 and 32kDa (Figure 3.8c). The 21 and 16kDa bands were

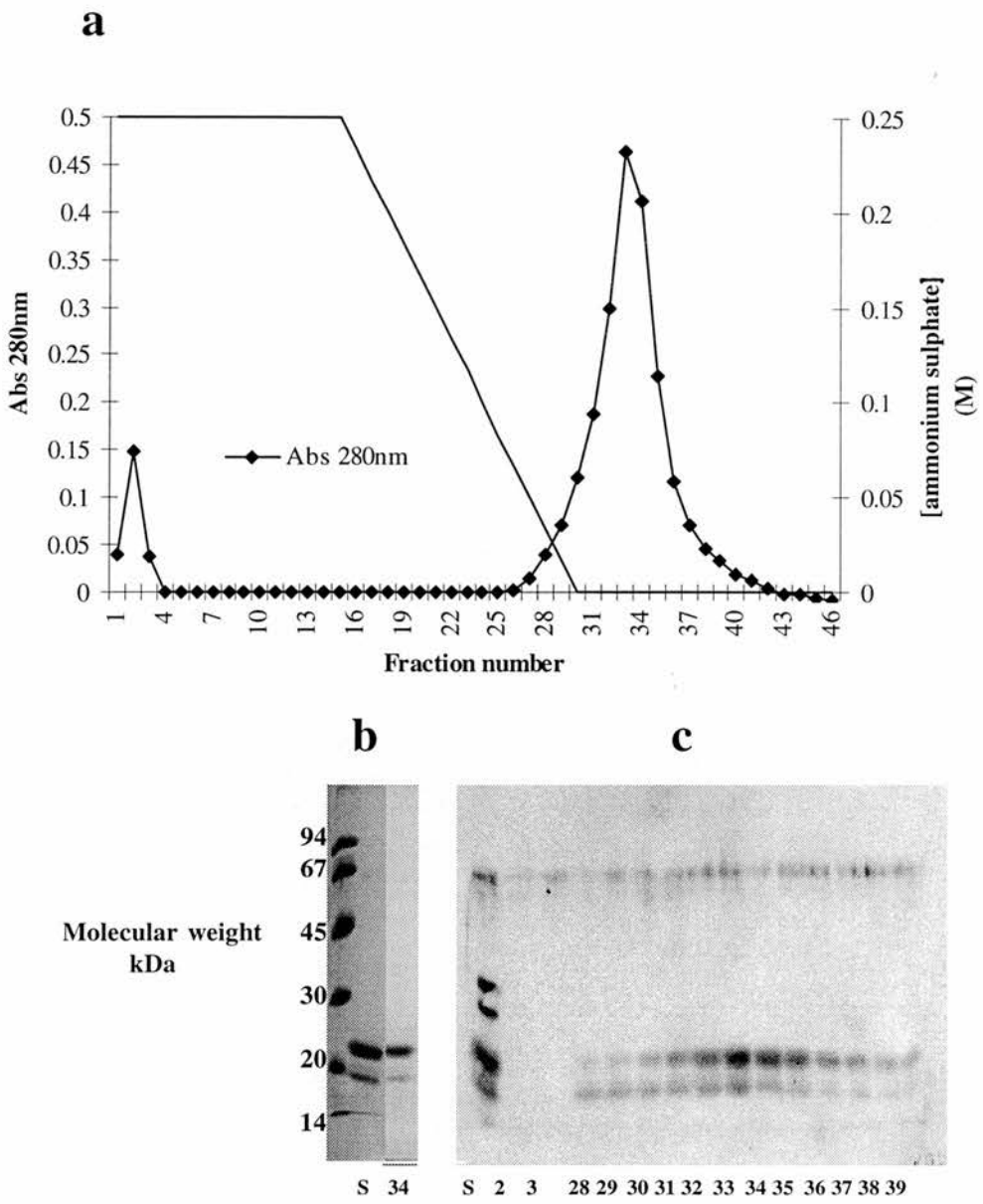


Figure 3.8. Separation of TRAMP and lysyl oxidase by octyl sepharose 4 fast flow hydrophobic interaction chromatography. Fractions from Superdex-75 containing both lysyl oxidase and TRAMP were loaded onto an octyl sepharose column in 50mM sodium phosphate, 0.25M ammonium sulphate, pH 7.0. (a) Proteins were eluted from the column with a gradient of 0.25M to 0M ammonium sulphate in 50mM sodium phosphate at a flow rate of 0.5ml/min. (b) SDS-PAGE and Coomassie blue staining of sample prior to loading (S) and peak fraction (34). (c) Western blot with antibody to lysyl oxidase (1:2500 dilution) of sample prior to loading (S) and peak fractions 2, 3 and 28 to 39.

ascribed to TRAMP and its degradation product (see discussion) whilst the 32 and 26kDa bands were believed to be lysyl oxidase and a degradation product of lysyl oxidase, respectively. No bands at 32kDa were observed in any fractions eluted from the octyl Sepharose column suggesting that lysyl oxidase either remained bound to or precipitated on the column. Attempts to repeat this separation procedure with mixtures of TRAMP and lysyl oxidase free from degradation products were unsuccessful. It should also be noted that TRAMP prepared in this way was inactive in the fibrillogenesis assays described in section 4.2.2 (data not shown).

3.4.8 Mono Q FPLC anion exchange chromatography

Mono Q FPLC anion exchange chromatography has previously been used as a final step in the purification of TRAMP to separate the 5 charge variants and to eliminate traces of lysyl oxidase (Cronshaw *et al.*, 1993).

Separation of the charge variants of TRAMP was performed by anion exchange on a Pharmacia Mono Q HR5/5 FPLC (Fast Protein Liquid Chromatography) column using a Gilson HPLC system at room temperature. Prior to this, buffer exchange was carried out on a Pharmacia Sephadex G-10 column pre-equilibrated with 6M TBU, pH 7.5 (20mM Tris-HCl, 6M urea). All buffers were made up using Milli-Q water and were filtered and degassed with 0.22μm filters just prior to use. The method of Macbeath *et al.* (1993) was used with the following modifications. Samples were loaded onto the Mono Q FPLC column, pre-equilibrated with 6M TBU, from a 1ml sample loop and were eluted with a linear gradient from 0 to 1M NaCl in 6M TBU at a flow rate of 1ml/min.

Figure 3.9a shows the elution profile obtained after Mono Q FPLC of TRAMP purified by gradient elution from DEAE-Sephacel followed by size exclusion on Superdex-75 column in the presence of urea. Four ionic variants were identified corresponding to variants T2 to T5 previously identified by Macbeath *et al.* (1993). Variant T1 was absent. A single 22kDa band was observed for each of these variants after SDS-PAGE followed by silver staining (Figure 3.9b).

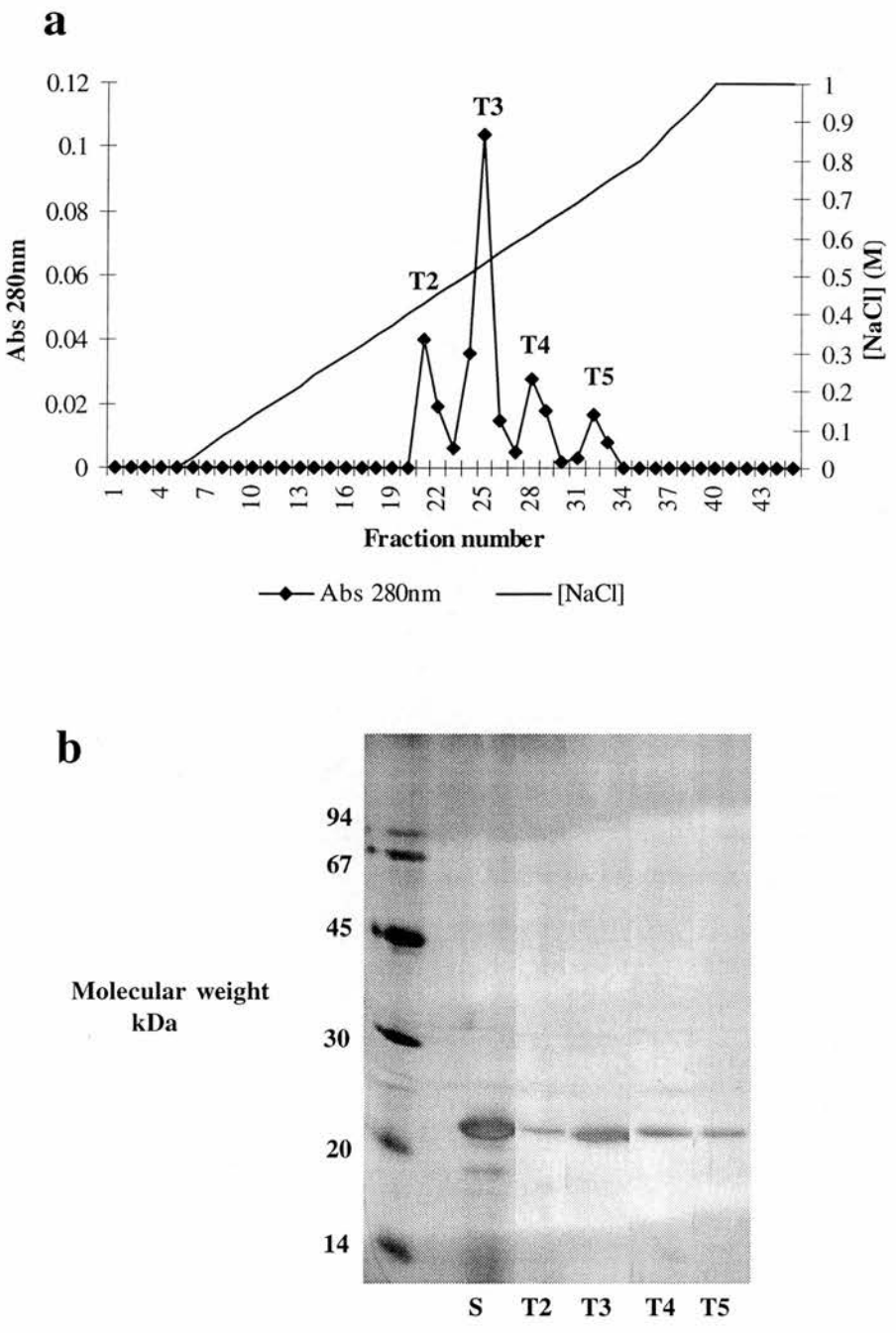


Figure 3.9. Separation of TRAMP variants by Mono Q FPLC. (a) Purified TRAMP was loaded on to the column from a 1ml sample loop in 10mM Tris-HCl, 6M urea, pH 7.5 and eluted with a gradient of 0 to 1M NaCl at a flow rate 1ml/min. (b) Peak fractions (T2, T3, T4 and T5) were analysed by SDS-PAGE followed by silver staining. S represents the sample prior to loading.

3.5 Discussion

DEAE-Sephacel anion exchange chromatography is a good initial technique for protein purification since it is able to process relatively large sample volumes. Macbeath *et al.* (1993) found that at pH 6.0, lysyl oxidase did not bind to the DEAE-Sephacel column and thus by running the column under these conditions substantial separation of TRAMP and lysyl oxidase could be achieved. However, in this study lysyl oxidase remained bound to the DEAE-Sephacel column at pH 6.0 and thus co-eluted with TRAMP. These contradictory results may be explained by differences in the loading conditions. Macbeath and co-workers adjusted the pH of the urea extract prior to loading onto the column, whilst in the present study the extract was loaded onto the column at pH 7.8 and the pH was subsequently lowered by washing the column with 10mM Bis-Tris, 6M urea, pH 6.0. Additionally, Macbeath *et al.* (1993) used phosphate buffer throughout the DEAE step as opposed to the Bis-Tris buffer used here.

Gradient elution from the DEAE-Sephacel column was more successful for the partial removal of both lysyl oxidase and other contaminating proteins. However, there were concerns about the effect of exposure of TRAMP to non-physiological pH conditions for prolonged periods. Thus, since little difference in the elution profile resulted from carrying out the gradient elution at pH 7.0, DEAE-Sephacel anion exchange chromatography at pH 7.0 with a gradient from 0 to 0.5M NaCl was judged to be a good initial technique for the removal of the bulk of contaminating proteins and partial separation of lysyl oxidase from TRAMP. Previously, four peaks of lysyl oxidase activity were observed during gradient elution from a DEAE anion exchange column corresponding to the 4 charge variants of lysyl oxidase (Stassen, 1976; Kagan *et al.*, 1979). However, in the present study only three peaks were observed suggesting that only three of the four variants bound to the column under the conditions used.

The basis of the interactions between both TRAMP and lysyl oxidase and Sephacryl S-200/S-400 is poorly understood. Kagan and Trackman (1991) suggested that lysyl oxidase formed aggregates in the absence of urea which were retained at the top of

the S-200 gel filtration column. Addition of urea would then lead to disaggregation and elution of lysyl oxidase. Alternatively, the interaction may be more specific. The separation of lysyl oxidase from TRAMP on a Sephacryl S-400 column could not be reproduced in the present study. Cronshaw *et al.* (1993) showed TRAMP to elute with 1.5M urea and lysyl oxidase with 6M urea whereas in these experiments both proteins only eluted after addition of 6M urea. It is unclear why such differences should occur.

Partial separation of TRAMP and lysyl oxidase was achieved by affinity chromatography on the amino hexyl Sepharose column although significant levels of high molecular weight contaminants were also present in fractions containing TRAMP. As expected, lysyl oxidase was found to bind to the amino hexyl Sepharose column although its affinity for the 1,6-diaminohexane column would seem to be lower than for bovine serum amine oxidase since octylamine was not required for its elution. More unexpectedly, TRAMP was also found to interact with the column. This could represent either a specific interaction with the 1,6-diaminohexane ligand or an interaction with the Sepharose 4B support. If the former was the case, this would support the hypothesis that TRAMP is also an amine oxidase (section 1.6.4.3).

TRAMP and lysyl oxidase could not be separated by preparative isoelectric focusing using the Rotofor cell (Biorad) due to the focusing of lysyl oxidase at a pH below its reported pI values. Previous pI values for lysyl oxidase (5.2- 5.8), obtained experimentally by Macbeath *et al.* (1993) and Cronshaw *et al.* (1993), were lower than the theoretical value of 6 calculated from the human lysyl oxidase sequence using GCG software. It is possible that this discrepancy is the result of post-translational modifications such as tyrosine sulphation. However, it is unclear why such discrepancies should occur between values obtained by analytical and preparative isoelectric focusing. Perhaps focusing in the Rotofor cell did not reach completion. Alternatively it may be the result of association of lysyl oxidase with TRAMP.

Superdex-75 size exclusion chromatography in the presence of urea was found to be the most promising method for the purification of TRAMP. Although separation of

TRAMP and lysyl oxidase was incomplete, the highest yields of TRAMP were obtained by this method (Table 3.2). It was also an efficient technique for removal of other contaminating proteins resulting in TRAMP of high purity. The speed and reproducibility of this technique was also in its favour.

Running the Superdex-75 column in the absence of urea resulted in complete separation of TRAMP from lysyl oxidase although yields were lower than in the presence of urea (Table 3.3). As is the case with Sephacryl S-200/S-400, both proteins interact with the Superdex-75 media, although the binding appears to be weaker since urea is not necessary for elution. The interaction with TRAMP is strongest and thus TRAMP is eluted with a K_{av} greater than 1, indicative of interaction of a protein with the size exclusion column. The results also appear to contradict previous reports that lysyl oxidase aggregates in the absence of urea (Jordan *et al.*, 1977) although small amounts of both lysyl oxidase and TRAMP were observed in the high molecular weight peak by western blotting.

Although hydrophobic interaction chromatography on the octyl Sepharose 4 fast flow column appeared to remove lysyl oxidase from TRAMP, the final yield of TRAMP was not substantially increased compared to separation by Superdex-75 alone (Table 3.2). This was believed to be due to precipitation of TRAMP in ammonium sulphate solutions although previously it was reported (Macbeath, 1992) that TRAMP precipitates at 30% saturation of ammonium sulphate which corresponds to a molarity of 1.25. The fact that TRAMP only bound to media with the strongest hydrophobic ligand attached (i.e. octyl Sepharose) is consistent with a protein with few hydrophobic moieties on its surface. The lack of activity of TRAMP in the fibrillogenesis assay after purification on the octyl Sepharose column may be the result of irreversible denaturation of the protein (Gagnon, 1996). The ability of the lysyl oxidase antibody to recognise TRAMP may be the result of non-specific binding due to the high amounts of TRAMP present in the sample relative to lysyl oxidase. Alternatively it may have been the result of cross reactivity due to sequence similarities discussed below.

The elution profile after Mono Q anion exchange chromatography was identical to that obtained by Macbeath *et al.* (1993) except that the first peak (T1) was absent. This was most probably the result of lack of binding of T1 to the DEAE-Sephacel column at low pH.

A common feature of purification methods performed in the absence of urea was the interaction of TRAMP with the chromatography media. TRAMP interacted with both Superdex and Sephacryl media and possibly with the Sepharose 4B support of the amino hexyl column. Additionally, TRAMP interacts with DEAE-Sephacel in the absence of urea (Cronshaw *et al.*, 1993). The requirement of urea for the disruption of the interactions suggests that they are hydrophobic. However, a more specific interaction with the polysaccharides within the matrices cannot be ruled out. This would have implications for the interaction of TRAMP with ECM components containing carbohydrate moieties, for example proteoglycans. Okamato *et al.* (1996) demonstrated such an interaction of TRAMP with decorin, though this appears to be via the protein core rather than the GAG chain (section 1.6.4).

The difficulties in separating TRAMP and lysyl oxidase may be explained by a specific interaction between them or by structural and perhaps functional similarities. Since previous immunoprecipitation studies (Forbes *et al.*, 1994) did not identify a TRAMP-lysyl oxidase complex, a structural similarity seems more likely. In addition to similar molecular weights, both TRAMP and lysyl oxidase are hydrophilic proteins with similar pI values. They also have a number of common features within their amino acid sequences including hydrophilic N-terminal regions, 2 distinct tyrosine rich domains and a hydrophobic C-terminal domain (Cronshaw, 1993). Whether their co-purification is also the result of functional similarities is less clear. Evidence discussed in chapter 5 refutes the suggestion that TRAMP, like lysyl oxidase, is an amine oxidase. Thus, the only functional similar between the 2 proteins to date is their ability to bind collagen fibrils (Macbeath *et al.*, 1993; Cronland *et al.*, 1985).

Most of the purification methods described in this chapter were able to partially separate TRAMP from lysyl oxidase. The purification was hampered by problems

with the identification of lysyl oxidase since at low levels it could not be identified on Coomassie blue or silver stained gels (Figures 3.2 and 3.5). Until an antibody to lysyl oxidase became available detection relied solely on the ultrafiltration assay. Additionally, since a specific assay for TRAMP was not available, direct comparison of yields was not feasible unless TRAMP was the only protein present after that particular purification step (Tables 3.2 and 3.3). Techniques which removed all other contaminants and gave the minimum of overlap between elution of TRAMP and lysyl oxidase were thus favoured. Other criteria which were considered were the speed and reproducibility of the method and also its possible denaturing effect on TRAMP. Thus the purification protocol of choice was DEAE-Sephacel anion exchange chromatography at pH 7.0 with a gradient elution, followed by Superdex-75 size exclusion chromatography in the presence of urea and finally Mono Q FPLC. The yield of 10μg/g wet weight starting material obtained by this method (Table 3.2) is low compared to the value of 25μg/g obtained by Okamato *et al.* (1996) for purification of the bovine equivalent of TRAMP from skin. The method of Okamato *et al.* (1996) involved both gel filtration and DEAE anion exchange followed by 2 reverse phase chromatography steps. The increased yields obtained by Okamato and co-workers may be explained by the use of 4M guanidine HCl in place of urea for the initial extraction procedure. However, it is not known whether the denaturing effect of guanidine HCl is reversible and thus whether TRAMP would still be active in the fibrillogenesis assay (Chapter 4).

Chapter 4

Interaction of TRAMP with collagen I and decorin

4.1 Purification of collagen I monomers

The purification of native monomeric collagen I from lathyritic rat skin (section 2.2) was judged to be successful according to the following criteria: (i) SDS-PAGE analysis showed the preparation to consist almost entirely of collagen α-chains, with the remaining 3% of protein bands comprising multimeric, cross-linked collagen β and γ components (Figure 4.1). (ii) Scanning densitometry of the Coomassie blue stained gel showed the ratio of α1 to α2 chains to be 2:1 suggesting that no collagen III was present. (Collagen III is present in skin and under reducing conditions the α1(III) chain migrates with a similar electrophoretic mobility to the α1(I) chain). (iii) The absence of collagen III was confirmed when identical gels were obtained under both reducing and non-reducing conditions (Figure 4.1). (Collagen III contains reducible disulphide bonds and therefore migrates more slowly under non-reducing conditions). (iv) No bands were detected after Alcian blue staining (section 2.3.2.3) showing that the preparation was free from contaminating proteoglycans. The yields of collagen I were high, with typically 400μg/g wet weight of starting material obtained.

The overall time required for the purification of monomeric collagen I from lathyritic rat skin, including the 3 week period in which rats were fed on the diet containing β-APN, was typically 6 weeks. In view of time constraints, a commercial supplier was investigated as a possible alternative source of collagen. Collagen I in 0.5M acetic acid was obtained from Coletica (courtesy of Dr. D. Herbaage) and found by SDS-PAGE analysis to have approximately equal ratios of α, β and γ components. The presence of large amounts of cross-linked components was judged to be unacceptable and therefore attempts were made to remove high molecular weight components by differential salt precipitation and fibril formation. Aliquots were taken and 20% (w/v) NaCl was slowly added to a final concentration of 3.5% (w/v) to precipitate out aggregates (Chandrakasen *et al.*, 1976). After centrifugation and dialysis of the supernatant to remove salt, the collagen was formed into fibrils overnight using the 'warm start' technique (section 4.2.2.1). Fibrils were pelleted by centrifugation at 15,600*g* for 10 mins and then extracted overnight in 1.1mM

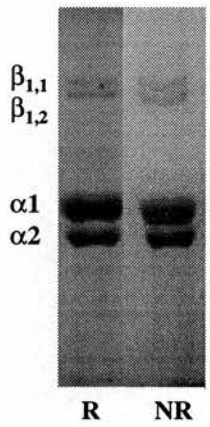


Figure 4.1. SDS-PAGE analysis of collagen I purified from lathyritic rat skin. Analysis by SDS-PAGE with 6% acrylamide in the separating gel under reducing (R) and non-reducing (NR) conditions. Proteins were visualised by Coomassie blue staining.

KH_2PO_4, 5.4mM Na_2HPO_4, 0.15M NaCl, pH 7.4. SDS-PAGE analysis of the extract showed no change in the ratio of α, β and γ components. Thus it proved impossible to eliminate the cross-linked components and the use of commercial collagen was abandoned.

4.2 Further characterisation of the TRAMP-collagen interaction

4.2.1 Materials

Hydrogen peroxide, Tween-20, bovine serum albumin, TES and o-phenyldiamine dihydrochloride were obtained from Sigma Chemicals co., Poole, Dorset. Normal goat serum and horse radish peroxidase conjugated goat anti-rabbit IgG antibody were obtained from the Scottish Antibody Production Unit (SAPU), Carluke, Lanarkshire. Decorin purified from bovine skin was a gift from Professor Paul Scott, University of Alberta, Edmonton, Canada. All other reagents, unless otherwise stated were obtained from BDH, Poole, Dorset.

4.2.2 Special methods

4.2.2.1 *In vitro* fibril formation assay

Lathyritic collagen I monomers (section 4.1) were reconstituted into fibrils using the "warm start technique" (Holmes *et al.*, 1986). Collagen monomers in 5mM acetic acid (250µl) and the appropriate double strength fibril formation buffer (250µl) were preincubated separately at 34°C before mixing in a 1:1 ratio to initiate fibril formation. The increase in turbidity was monitored at 313nm at 5 min intervals in a Philips/Unicam 8700 series UV/VIS spectrophotometer. Data was transferred to Microsoft Excel using Unicam ASDS software. For fibril formation in the presence of TRAMP or decorin, solutions containing these proteins were subjected to buffer exchange on 1ml Biogel P-6DG (Biorad; section 3.3), previously swollen and equilibrated with the double strength fibril formation buffer to be used, and then diluted in the same buffer prior to use.

Although collagen solutions were centrifuged (300,000*g*, 1 hour, 4°C) just prior to use, and all other solutions were filtered with 0.22µm Millipore filters, initial experiments gave large sample to sample variations. These variations were minimised by rinsing all tubes and tips with filtered buffers, using transmission matched quartz cuvettes with lids (Hellma) rather then plastic disposable cuvettes and including a final concentration of 100µg/ml BSA in the cuvette. The presence of BSA is believed to prevent non-specific absorption and protein denaturation due to interactions with walls of tubes and pipette tips (Brown and Vogel, 1989).

4.2.2.2 Binding assays

4.2.2.2.1 Co-sedimentation assay

To study the interaction of TRAMP with collagen I fibrils under various conditions, fibrils were formed as described above. The insoluble fibrils were pelleted by centrifugation at 15,600*g* at room temperature for 10 mins. The supernatant was removed and proteins were precipitated by the addition of TCA to a final concentration of 10% (w/v) followed by incubation on ice for 15 mins, then centrifugation at 15,600*g* for 10 mins. Both fibril and TCA pellets were redissolved in reducing sample buffer (Hames, 1990) and analysed by SDS-PAGE, Coomassie staining and densitometry (section 2.3). To quantify the amounts of TRAMP found in the pellet and supernatant, standards containing known amounts of TRAMP were run on the same gel to give a standard curve of µg of TRAMP versus optical density. To measure the amounts of collagen in the supernatant and pellet, parallel fibrillogenesis assays were set up and pellets were redissolved in 0.5M acetic acid and assayed using the Sircol collagen assay (section 2.6.2.1) or by SDS-PAGE followed by densitometry. The reproducibility was improved by the inclusion of 100µg/ml BSA in the buffer to prevent protein losses.

4.2.2.2.2 Solid phase assay

A modification of the ELISA method of Hedbom and Heinegard (1989) was used to measure the binding of TRAMP to collagen I monomers immobilised on microtiter plate wells.

The wells of a polystyrene microtiter plate (Nunc-Immuno Plate IF, article no. 439454, AS Nunc) were coated with 200µl of soluble collagen I (5µg/ml), obtained by mixing equal volumes of collagen in 5mM acetic acid and PBS1 (0.27M NaCl, 60mM sodium phosphate, pH 7.4) at 4°C. Control wells were coated with the same concentration of BSA. The plate was incubated at 4°C overnight in a humidity chamber before crosslinking absorbed protein by treatment with 0.25% (v/v) glutaraldehyde in 0.15M NaCl, 0.5mM $NaCNBH_4$, 20mM TES, pH 7.4 for 2.5 hours at 37°C. The remaining reactive groups were then blocked by incubating wells for 30 mins at 37°C with 0.2M ethanolamine in the same buffer. After washing with 5 x 200µl of PBS2 (0.14M NaCl, 5mM sodium phosphate, 0.05% Tween-20, pH 7.4), non-specific binding was blocked with 200µl/well of PBS3 (PBS2 containing 100mg/ml BSA) for 4 hours at room temperature. Wells were washed with 5 x 200µl PBS4 (0.14M NaCl, 30mM sodium phosphate, 0.05% Tween-20, pH 7.4) and incubated with 200µl TRAMP in PBS4 overnight at room temperature with gentle shaking. The washing step was repeated and 200µl of anti-TRAMP antibody was added to each well and incubated overnight at 4°C. The plate was again washed before incubation with 200µl/well of a 1:5000 dilution of horse radish peroxidase (HRP) conjugated goat anti-rabbit IgG antibody for 2 hours at room temperature. A final washing step was followed by incubation in the dark for 40 mins with 200µl/well 0.4mg/ml o-phenyldiamine dihydrochloride (OPD) in phosphate-citrate buffer (0.05M Na_2HPO_4, 0.024M citric acid, pH 5.0) containing 0.4µl/ml H_2O_2. The reaction was stopped with the addition of 50µl 3M sulphuric acid and the absorbance at 490nm was measured in a Dynatech MR7000 microplate reader. All experiments were carried out in triplicate and controls were included in the absence of collagen or TRAMP.

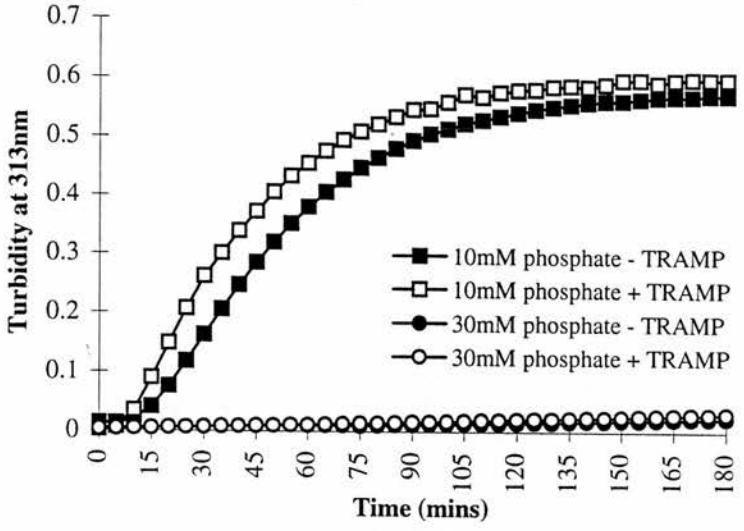
4.2.3 Effect of buffer system on interaction with fibrils

As discussed previously, TRAMP interacts with collagen I fibrils reconstituted *in vitro* and accelerates their formation in 30mM Na_2HPO_4, 30mM TES, 135mM NaCl, pH 7.4 at 34°C (section 1.6.4.2; Macbeath *et al.*, 1993). This buffer produces fibrils which are morphologically identical to fibrils observed *in vivo* (Williams *et al.*, 1978). However, the concentrations of phosphate and TES in this buffer are not compatible with physiological conditions. Also, both phosphate and TES concentrations are known to affect the kinetics of fibril formation *in vitro* (Williams *et al.*, 1978; Pogany *et al.*, 1994). It was thus pertinent to study the effect of phosphate and TES concentrations on the ability of TRAMP to accelerate collagen fibril formation and on the binding of TRAMP to these fibrils.

4.2.3.1 Kinetics of fibril assembly

The effect of phosphate and TES concentration on the ability of a mixture of TRAMP variants (T2 to T5) to accelerate fibril formation was assessed using the fibril formation assay (section 4.2.2.1) with a final concentration of 100µg/ml collagen. Fibril formation was almost completely inhibited in TES buffer (30mM TES, 135mM NaCl, pH 7.4) containing 30mM phosphate both in the presence and absence of 5µg/ml TRAMP (Figure 4.2a). By comparison, initiation of fibril formation in TES buffer containing 10mM phosphate in the absence of TRAMP resulted in a characteristic sigmoidal turbidity-time curve (Figure 4.2a; section 1.7; Figure 1.3). This observation is in agreement with previous reports showing that increasing phosphate concentrations have an inhibitory effect on the kinetics of fibril formation (Williams *et al.*, 1978; Pogany *et al.*, 1994). Figure 4.2a also shows that the acceleratory effect of TRAMP on fibril formation, characterised by a decrease in the lag phase and an increase in final turbidity, is still observed in 10mM phosphate. Figure 4.2b demonstrates the effect of TES on fibril formation in the presence and absence of 5µg/ml TRAMP in 10mM PBS buffer (7.7mM Na_2HPO_4, 2.3mM NaH_2PO_4, 135mM NaCl, pH 7.4). In the presence of 30mM TES the lag phase was ~5 mins, $t_{1/2}$ was 16 mins and the final turbidity was 0.358. In contrast, when fibrils

(a) Effect of [phosphate]



(b) Effect of [TES]

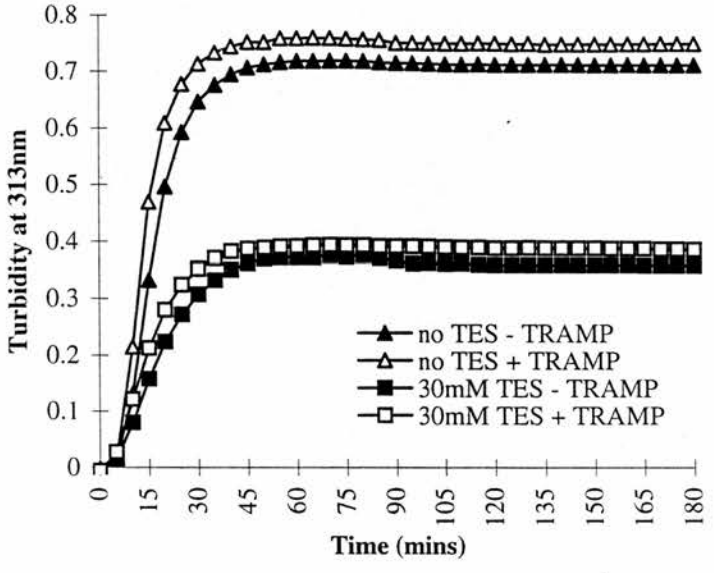


Figure 4.2. Effect of phosphate and TES concentration on collagen fibril formation *in vitro*. Fibril formation was intiated using the 'warm start' technique. After preincubation at 34°C, collagen I in 5mM acetic acid and double strength buffer were mixed in a 1:1 ratio to give a final [collagen] of 100µg/ml. The increase in turbidity at 313nm was monitored every 5 mins for 180 mins. All buffers contained 135mM NaCl. Appropriate phosphate buffer (PB) concentrations were achieved by diluting 60mM PB (46.4mM Na_2HPO_4, 13.6mM NaH_2PO_4, pH 7.4). For fibrils formed in the presence of TRAMP, the final [TRAMP] was 5µg/ml. Total sample volume in the cuvette was 0.5ml.

were formed in the absence of TES, $t_{1/2}$ was decreased to 14 mins and the final turbidity was increased to 0.711. The change in lag phase could not be accurately measured. The inhibitory effect of TES on collagen fibril formation is in agreement with previous reports (Pogany *et al.*, 1994). Figure 4.2b also shows that TRAMP was still able to accelerate fibril formation in the absence of TES judged by a decrease in $t_{1/2}$ to 9 mins and an increase in final turbidity to 0.749.

4.2.3.2 Binding to fibrils

Collagen fibrils were formed overnight at 34°C at various phosphate and TES concentrations with 100µg/ml collagen and 5µg/ml TRAMP and the effect on the binding of TRAMP to fibrils was assessed using the co-sedimentation assay described in section 4.2.2.2.1 (Table 4.1). In TES buffer (30mM TES, 135mM NaCl, pH 7.4) containing 30mM phosphate, 7% of the total TRAMP present was found in the pellet. When the phosphate concentration was reduced to 10mM the amount of TRAMP in the pellet was increased to 12% of the total. Removal of TES from this buffer resulted in a further increase to 21%. It appears therefore that both phosphate and TES have an inhibitory effect on TRAMP binding to collagen. However, differences in the composition of the fibril formation buffer also affect the amount of collagen formed into fibrils, hence the ratios of TRAMP to collagen found in the supernatant and the pellet were also compared under the different conditions. The ratio of TRAMP to collagen was decreased when the phosphate concentration was lowered to 10mM suggesting that less TRAMP was associated with collagen at lower phosphate concentrations. In contrast, removal of TES from buffer containing 10mM PB, 135mM NaCl, pH 7.4 appeared to have no further affect on the ratio of TRAMP to collagen and thus on the amount of TRAMP associated with collagen fibrils.

4.2.3.3 Binding of TRAMP to collagen fibrils after formation

Previous studies on binding of TRAMP to *in vitro* reconstituted collagen fibrils looked only at the ability of TRAMP to bind fibrils as they form (Macbeath *et al.*,

	μg TRAMP		μg collagen I		Ratio of TRAMP: collagen I	
	P	S	P	S	P	S
30mM TES, 30mM PBS, pH 7.4	0.183 ±0.069	2.385 ±0.378	5.385 ±3.217	38.156 ±3.188	0.028-0.053:1	0.057-0.067:1
30mM TES, 10mM PBS, pH 7.4	0.302 ±0.077	2.383 ±0.064	21.205 ±1.247	30.357 ±0.762	0.011-0.017:1	0.078-0.079:1
10mM PBS, pH 7.4	0.526 ±0.096	2.314 ±0.351	33.930 ±1.804	27.285 ±0.068	0.013-0.017:1	0.072-0.097:1

Table 4.1. The effect of phosphate and TES concentration on TRAMP binding to collagen I fibrils. Amounts of TRAMP (determined by scanning densitometry) and collagen (determined by sircol assay) in pellets (P) and supernatants (S) after centrifugation of fibrils formed at 34°C for 16 hours in various buffer systems. Reaction volume was 500μl. All buffers contained 135mM NaCl and appropriate phosphate buffer (PB) concentrations were achieved by diluting 60mM PB (46.4mM Na_2HPO_4, 13.6mM Na_2HPO_4, pH 7.4). Data represent means ± standard errors (n=4).

1993). Since TRAMP is postulated to have a role in the initial nucleation phase of fibrillogenesis, experiments were devised to study the binding of TRAMP to fibrils previously formed in the absence of TRAMP. Fibril formation was initiated in Eppendorf tubes using the 'warm start' technique (section 4.2.2.1) in 30mM TES, 30mM Na_2HPO_4, 135mM NaCl, pH 7.4 with 600µg/ml collagen in a final volume of 250µl. After allowing fibrils to form for 20 hours at 34°C, 250µl of the same buffer containing 10µg/ml TRAMP was carefully layered on top of the collagen gel followed by further incubation for 20 hours at 34°C. The amount of TRAMP associated with fibrils was assessed using the co-sedimentation assay (section 4.2.2.2.1). The results show that TRAMP does not associate with collagen I fibrils if not present during their formation (Figure 4.3). Furthermore, no TRAMP was found associated with fibrils formed in the presence of 10µg/ml TRAMP and then layered with buffer alone. In contrast, TRAMP was still found associated with collagen fibrils after a 40 hours incubation period if the mixture was not layered with buffer (Figure 4.3).

4.2.4 Effect of sulphatase-treated TRAMP on the kinetics of fibril assembly

TRAMP is known to contain sulphated tyrosine residues (section 1.6.2; Forbes *et al.*, 1994). To determine whether sulphation was involved in the acceleratory effect of TRAMP on fibril assembly, sulphate groups were partially removed by treatment with aryl sulphatase.

For sulphatase treatment, 275µg of TRAMP in 0.1M sodium acetate, pH 5.0 was incubated with 1.25 units of aryl sulphatase sulphohydrolase from *Helix pomatia* (E.C. 3.1.6.1; Boehringer Mannheim) for 2 hours at 37°C in a total volume of 1ml (Forbes *et al.*, 1994). TRAMP was then separated from the sulphatase by size exclusion chromatography on a Sephadex G50 column (1.3 x 30cm) pre-equilibrated with TES buffer (60mM TES, 60mM Na_2HPO_4, 270mM NaCl, pH 7.4). For controls, TRAMP was incubated in 0.1M sodium acetate for 2 hours at 37°C in the absence of sulphatase, before buffer exchange on the Sephadex G50 column. Control and

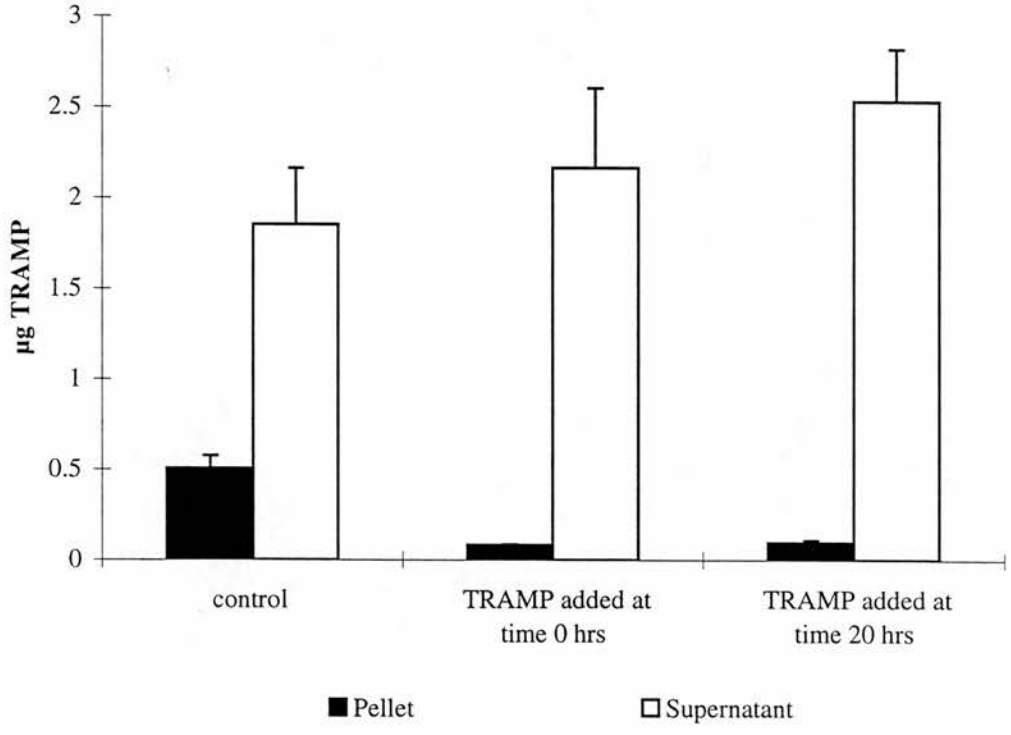


Figure 4.3. Comparison of binding of TRAMP to fibrils as they form and after formation. Collagen fibrillogenesis was initiated at time 0 hrs in the presence or absence of 2.5µg TRAMP. At time 20 hrs, buffer or TRAMP was layered over the collagen gel and incubated for a further 20 hrs before analysis using the co-sedimentation assay. In controls, collagen fibrils were formed in the presence of TRAMP and incubated undisturbed for 40 hrs at 34°C. Error bars represent standard errors (n=2).

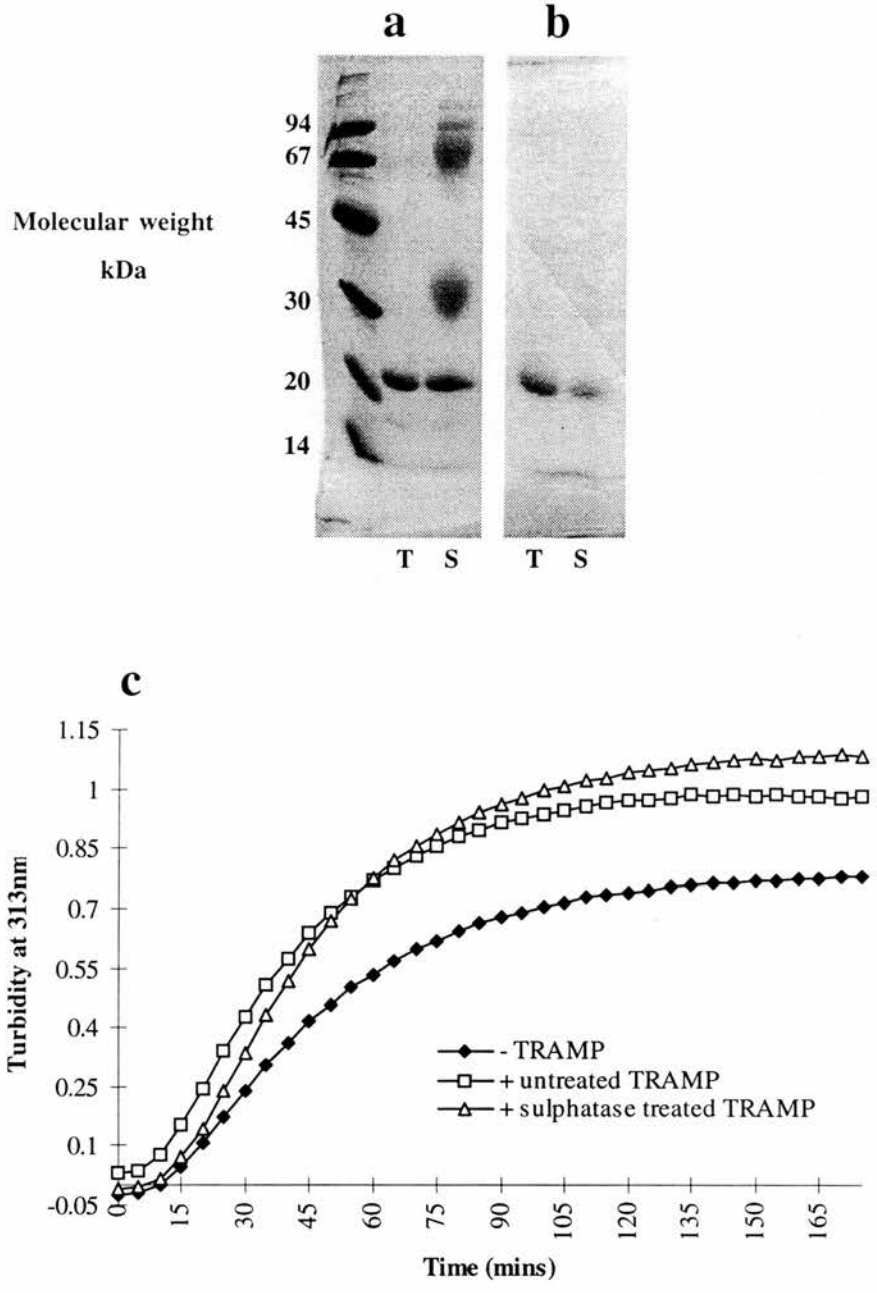


Figure 4.4. Effect of partial removal of sulphate groups from TRAMP on collagen fibril formation *in vitro*. For sulphatase treatment, TRAMP was incubated with 1.25 units of sulphatase for 2 hours at 37°C in 0.1M sodium acetate, pH 5.0. Samples before (T) and after (S) sulphatase treatment were analysed by SDS-PAGE followed by Coomassie (a) or Alcian (b) blue staining. In lane S diffuse bands at ~67kDa and ~32kDa correspond to sulphatase and its degradation product. (c) Fibrils were formed at 34°C in 30mM TES, 30mM Na_2HPO_4, 135mM NaCl, pH 7.4 with 300µg/ml collagen and when present, 10µg/ml TRAMP. Total volume in cuvette was 0.5ml.

sulphatase-treated TRAMP were then tested in the fibril formation assay (section 4.2.2.1).

Samples of TRAMP before and after sulphatase treatment were subjected to SDS-PAGE, stained with either Coomassie or Alcian blue and analysed by densitometry. Alcian blue staining of TRAMP was reduced by 50% relative to Coomassie blue staining after sulphatase treatment (Figure 4.4a,b) which is consistent with previous reports (Cronshaw *et al.*, 1993). Thus, approximately half of the sulphate groups on TRAMP were removed by sulphatase treatment. Figure 4.4c shows that the partial removal of sulphate groups had no affect on the ability of TRAMP to accelerate fibril formation *in vitro*.

4.2.5 Interaction with monomers

The interaction of TRAMP with collagen monomers has not previously been studied in detail, though preliminary results using fluorescence polarisation and rotary shadowing indicate that such interactions may occur (Macbeath, 1992).

Initial experiments using the solid phase assay (section 4.2.2.2.2) showed clear binding of TRAMP (0.5µg/well) to collagen coated wells (1µg/well) using a 1:5000 dilution of primary antibody (Figure 4.5). However, high levels of non-specific binding were observed in the absence of TRAMP. This high background was reduced by using the antibody after affinity purification on a TRAMP-nitrocellulose strip as described in section 2.7 (Figure 4.5).

The binding capacity of TRAMP for collagen coats was investigated by titration of a fixed amount of collagen (1µg/well) with increasing concentrations of TRAMP (Figure 4.6). TRAMP binding increased rapidly up to 2.5µg/ml TRAMP and then began to level off towards saturation.

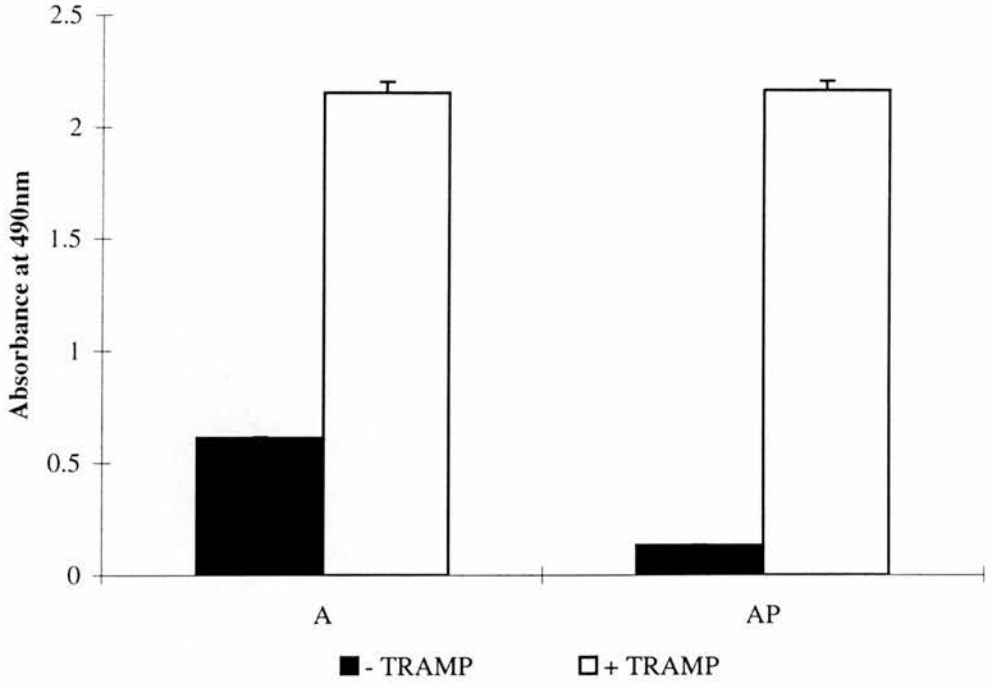


Figure 4.5. Effect of affinity purification on antibody specificity. Microtiter plate wells coated with 5µg/ml collagen were incubated overnight at room temperature with PBS4 alone or PBS4 containing 2.5µg/ml TRAMP and then incubated with antiserum to TRAMP before (A, 1:5000 dilution) or after (AP, 1:10 dilution) affinity purification. Controls were included in which wells were coated with 5µg/ml BSA in place of collagen and data is expressed after subtraction of control values. Error bars represent standard errors (n=3).

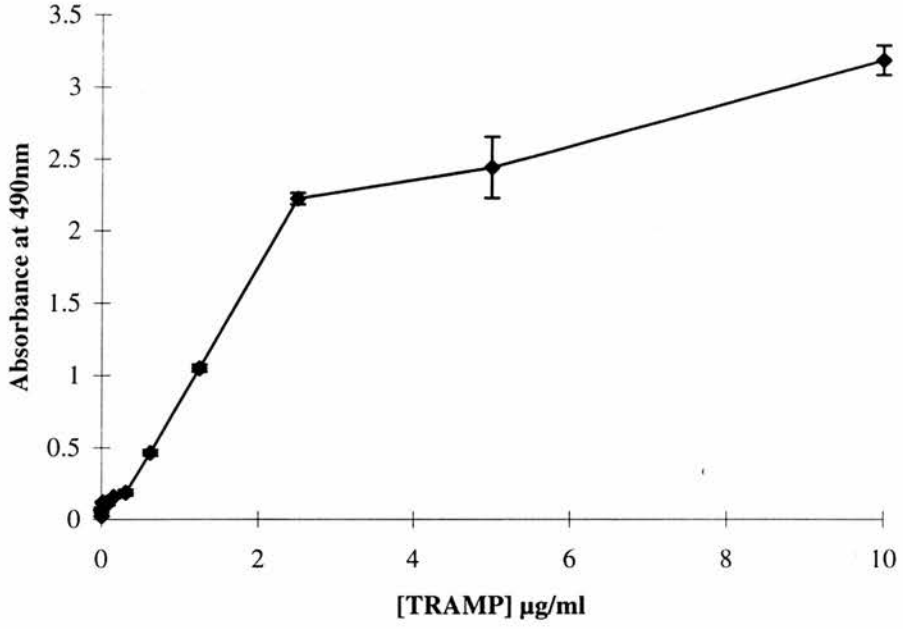


Figure 4.6. The binding capacity of TRAMP for collagen I monomers. Microtiter plate wells coated with 5µg/ml collagen were incubated at room temperature for 16 hours with various concentrations of TRAMP in PBS4. Bound protein was quantitated by ELISA as described in section 4.2.2.2.2 using a 1:20 dilution of affinity purified antiserum and a 1:5000 dilution of anti-rabbit IgG antibodies conjugated to horse radish peroxidase. A control experiment was also carried out in which wells were coated with 5µg/ml BSA before incubation with increasing concentrations of TRAMP and data is expressed after subtraction of these control values. Error bars represent standard error (n=3).

4.3 Effect of decorin on the TRAMP-collagen interaction

The ability of the small dermatan sulphate proteoglycan decorin to bind collagen I and inhibit collagen fibril formation *in vitro* is well documented (section 1.7.3; Vogel *et al.*, 1984). More recently, decorin has been shown to bind 22K ECM protein, the bovine form of TRAMP (section 1.6.4.2; Okatamo *et al.*, 1996). It was therefore of interest to study the effect of decorin on the TRAMP-collagen interaction.

4.3.1 Interaction with fibrils

4.3.1.1 Kinetics of fibril assembly

The effect of decorin on collagen fibril formation in the presence and absence of TRAMP was studied using the 'warm start' technique (section 4.2.2.1) in 30mM TES, 30mM Na_2HPO_4, 135mM NaCl, pH 7.4 (Figure 4.7). The characteristic turbidity-time curve was observed when fibrils were formed in the presence of 300µg/ml collagen (lag phase 9 mins; $t_{1/2}$ 40 mins; final turbidity 1.032). As expected, the presence of 5µg/ml decorin alone inhibited fibril formation, judged by increases in the lag phase (22 mins) and $t_{1/2}$ (65 mins) and a decrease in the final turbidity (0.765). Fibril formation was accelerated in the presence of TRAMP alone; the lag phase and $t_{1/2}$ were decreased to 6 mins and 23 mins respectively and the final turbidity was increased to 1.269. When both decorin and TRAMP were present, the effect of decorin predominated, although TRAMP partially reversed decorin inhibition. The lag time (17 mins) and $t_{1/2}$ (79 mins) were closest to the values obtained for decorin alone, but the final turbidity tended towards the control value. Taking the molecular masses of TRAMP and decorin to be 22kDa and 120kDa respectively, the molar ratio of TRAMP to decorin was 22 to 1. Thus, the inhibitory effect of decorin predominates even when present at much lower molar amounts.

The combined effect of decorin and TRAMP on collagen fibrillogenesis was also studied in 10mM PBS, pH 7.4 (Figure 4.8). When present alone, TRAMP accelerated and decorin inhibited fibrillogenesis although the effects of both were less

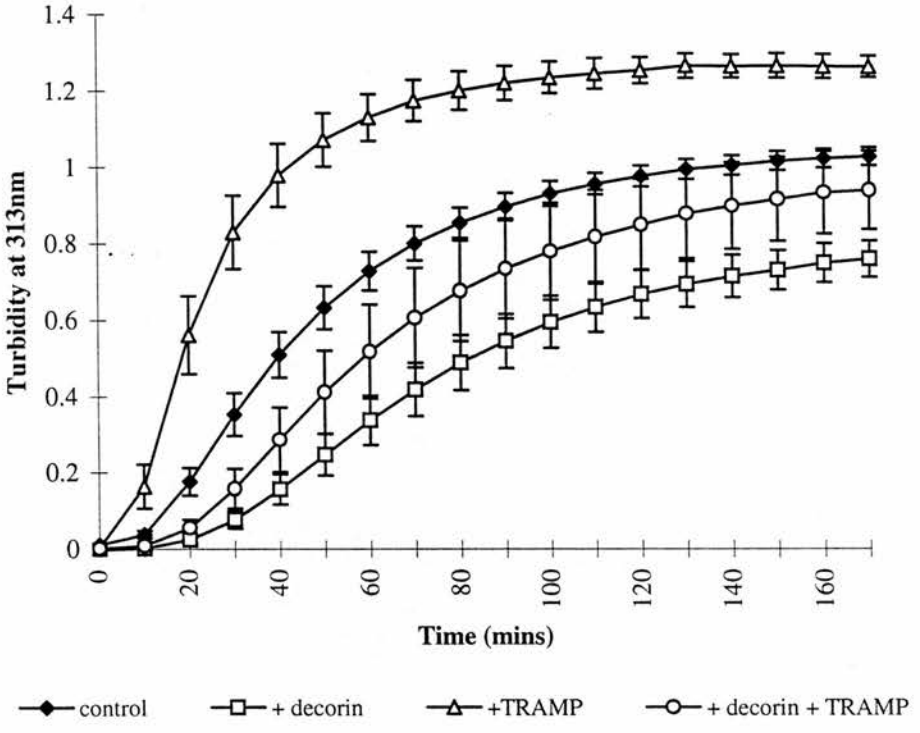


Figure 4.7. The combined effect of TRAMP and decorin on collagen fibril formation in TES buffer. Fibrils were formed at 34°C in 30mM TES, 30mM Na_2HPO_4, 135mM NaCl, pH 7.4 with 300µg/ml collagen and when present 5µg/ml decorin and 20µg/ml TRAMP. Total sample volume in the cuvette was 0.5ml. Error bars represent standard errors (n=4).

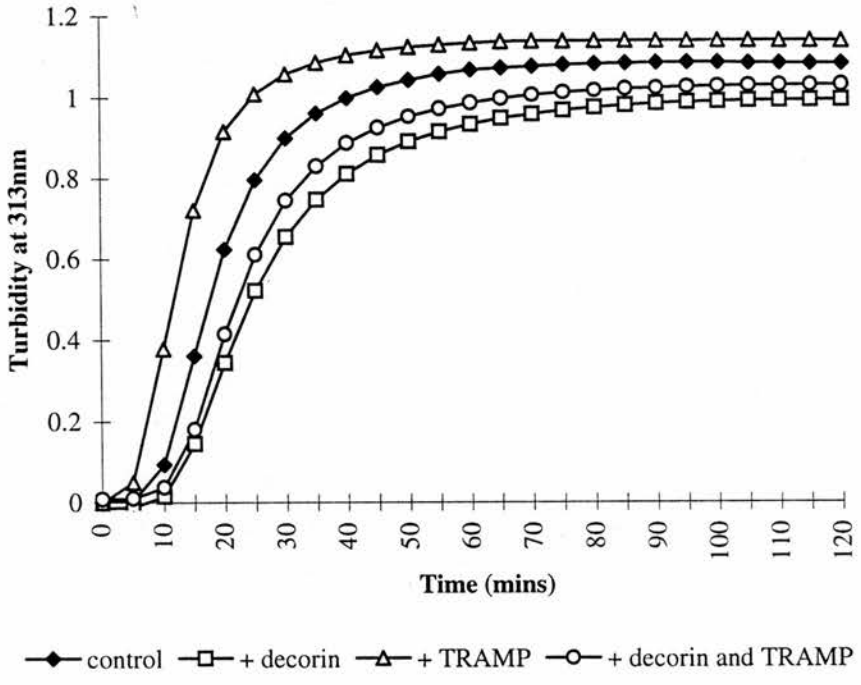


Figure 4.8. The combined effect of TRAMP and decorin on collagen fibril formation in PBS. Fibrils were formed at 34°C in 10mM PBS (7.7mM Na_2HPO_4, 2.3mM NaH_2PO_4, 135mM NaCl, pH 7.4) with 150µg/ml collagen and when present 2.5µg/ml decorin and 10µg/ml TRAMP. Total sample volume in the cuvette was 0.5ml.

pronounced than in TES buffer. When both TRAMP and decorin were present the decorin effect was again dominant.

4.3.1.2 Binding to fibrils

The effect of decorin on TRAMP binding to fibrils was studied using the co-sedimentation assay (section 4.2.2.2.1). When fibril formation was initiated in TES buffer with 300μg/ml collagen in the presence of 20μg/ml TRAMP, 64% of the collagen present was found in the pellet and thus formed into fibrils. By contrast, only 24% of collagen present was formed into fibrils in the presence of both 20μg/ml TRAMP and 5μg/ml decorin. Despite the decrease in collagen in the pellet, decorin appeared to have no effect on the amount of TRAMP found in the pellet (Figure 4.9). After incubating a mixture of 20μg/ml TRAMP and 5μg/ml decorin in the absence of collagen for 3 hours at 34°C, no TRAMP was found in the pellet (data not shown) suggesting that the presence of decorin does not cause precipitation of TRAMP. Thus, it would seem that decorin enhances TRAMP binding to collagen fibrils.

The effect of decorin on TRAMP binding to fibrils formed in 10mM PBS, pH 7.4 was then studied (Figure 4.10). Again, no change in the amount of TRAMP found in the pellet was observed in the presence of decorin. However, there was also little difference in the amount of collagen formed into fibrils in the presence and absence of decorin. Thus, in 10mM PBS, pH 7.4 decorin has no effect on TRAMP binding to collagen fibrils.

4.3.2 Interaction with monomers

The effect of decorin on TRAMP binding to collagen monomers was investigated using the solid phase assay (section 4.2.2.2.2). Firstly, TRAMP and various concentrations of decorin were added simultaneously and incubated for 24 hours in collagen coated wells (Figure 4.11). A small decrease in binding was observed with increasing decorin concentration. However, the presence of increasing concentrations of BSA resulted in a large decrease in binding suggesting that the presence of free

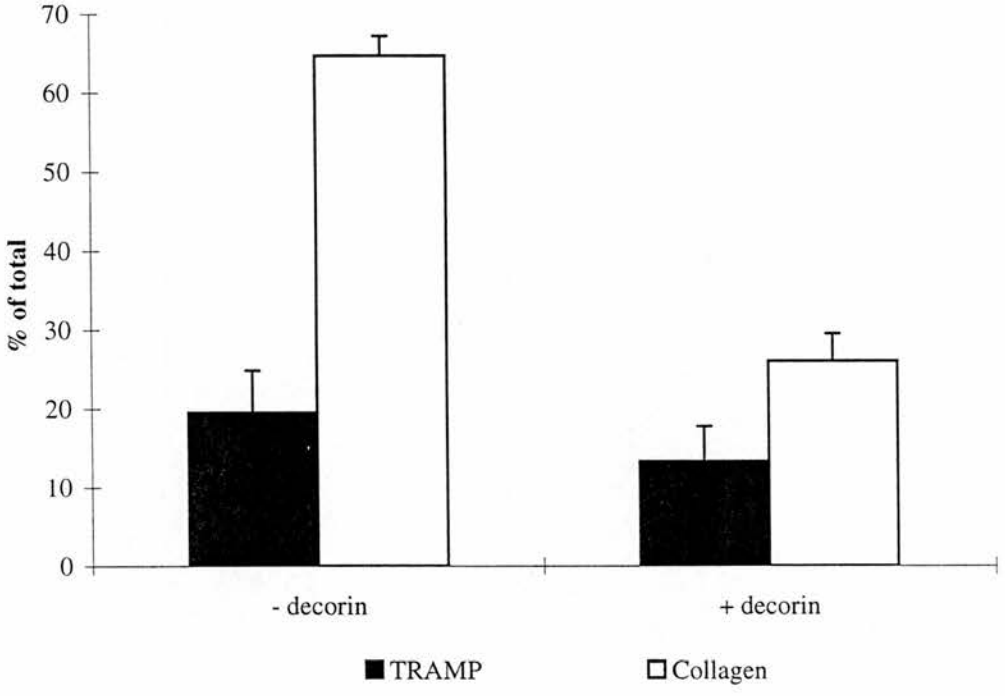


Figure 4.9. The effect of decorin on binding of TRAMP to collagen fibrils formed in TES buffer. Amounts of collagen and TRAMP in the pellet (determined by scanning densitometry) after centrifugation of fibrils formed for 3 hours with 300μg/ml collagen, 20μg/ml TRAMP and in the presence or absence of 5μg/ml decorin. Results are expressed as percentage of total collagen and TRAMP present. Error bars represent standard errors (n=3).

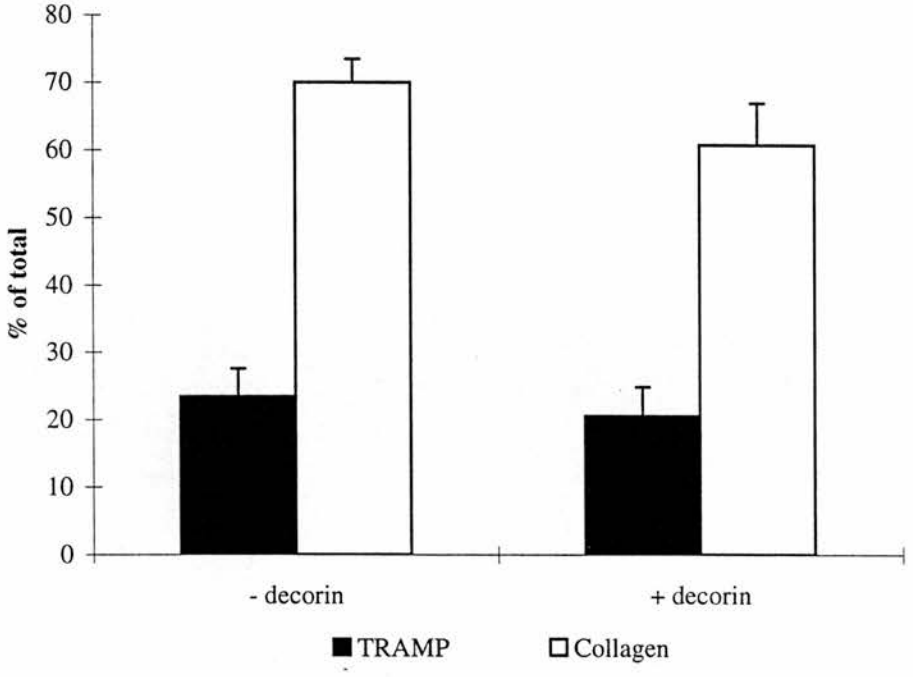


Figure 4.10. The effect of decorin on the binding of TRAMP to collagen fibrils formed in PBS. Amounts of collagen and TRAMP in the pellet (determined by scanning densitometry) after centrifugation of fibrils formed for 3 hours with 150μg/ml collagen, 10μg/ml TRAMP and in the presence or absence of 2.5μg/ml decorin. Results are expressed as percentage of total collagen and TRAMP present. Error bars represent standard errors (n=2).

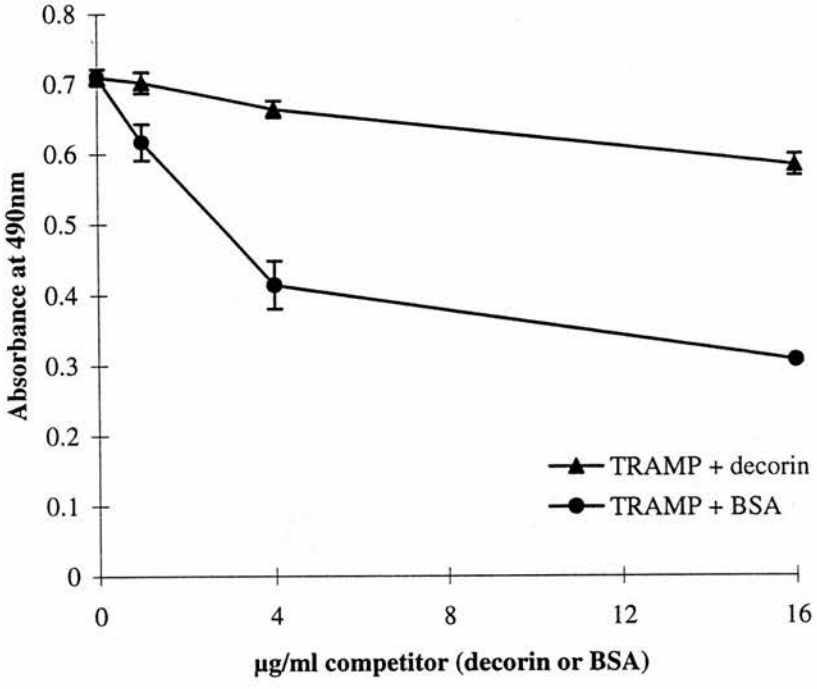


Figure 4.11. Binding of TRAMP to collagen I monomers in the presence of free decorin. Wells coated with 5µg/ml collagen were incubated with a mixture of 1µg/ml TRAMP and increasing concentrations of decorin or BSA for 20 hours. Amount of TRAMP bound was measured using an ELISA with a 1:20 dilution of affinity purified primary antibody. Error bars represent standard errors (n=2).

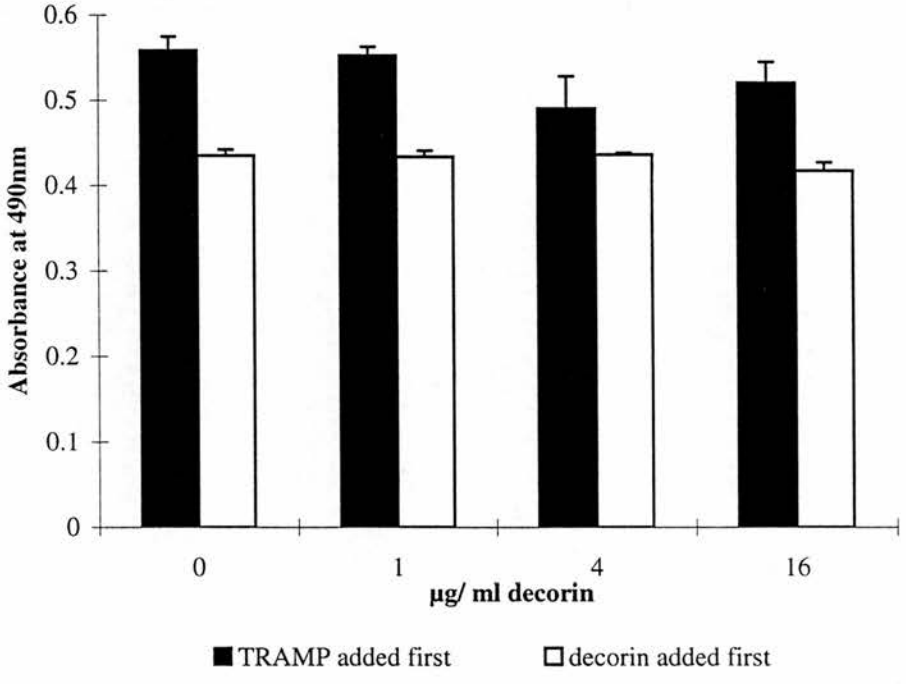


Figure 4.12. Effect of decorin on binding of TRAMP to collagen I monomers. Wells coated with 5µg/ml collagen were incubated with 1µg/ml TRAMP overnight followed by various concentrations of decorin for 8 hours (■) or with various concentrations of decorin overnight followed by 1µg/ml TRAMP for 8 hours (□). Amount of TRAMP bound was quantified by ELISA with a 1:20 dilution of affinity purified primary antibody. Absorbance values are expressed after subtraction of blanks with no TRAMP present. Error bars represent standard errors (n=2).

protein in the buffer interferes with the TRAMP-collagen interaction. Thus, in order to eliminate the effect of free protein, experiments were set up whereby TRAMP was allowed to bind to the collagen coat for 16 hours before incubating for a further 8 hours with decorin at various concentrations (Figure 4.12). In other cases, decorin was incubated with the collagen coat prior to incubation with TRAMP (Figure 4.12). Decorin had no affect on binding of TRAMP in either of these cases even when present at high saturable concentrations of 16μg/ml (Hedbom and Heinegard, 1989). This suggests that the binding sites for TRAMP and decorin on collagen are distinct. However, there did appear to be a decrease in binding when decorin was added prior to TRAMP. Since the effect was also observed in control wells where no decorin was added, the decrease is most likely to be due to the shorter incubation time with TRAMP in the second experiment.

4.4 Identification of TRAMP binding sites on collagen fibrils

4.4.1 Immunogold Labelling Technique

The original method used for indirect immunolabelling was based on that of Mould *et al.* (1990). Collagen fibrils reconstituted in the presence or absence of TRAMP were absorbed onto formvar, carbon coated nickel grids (Agar). Non-specific binding was blocked by floating grids face down on 50μl droplets of PBST (PBS containing 0.05% Tween-20) for 15 mins. Excess liquid was drained off and the grids were incubated for 2 hours with the appropriate dilution of TRAMP anti-serum diluted in PBST. Grids were washed on 5x 50μl droplets of PBST for 5 mins each before incubating for 1 hour with a 5nm gold-conjugated goat anti-rabbit probe diluted in PBST. The grids were washed on 5x 50μl droplets of dH_2O adjusted to pH 8.0 with NaOH. Finally the grids were positively stained with 1% (w/v) phosphotungstic acid, pH 2.2 followed by 1% (w/v) uranyl acetate, pH 4.4. Electron micrographs were analysed using a Semper image analysis programme to measure the distribution of gold particles along the fibril. Controls were included with preimmune serum or PBST alone in place of primary antibody. For incubations over 15 mins, grids were

placed in a humidity chamber. Prior to use, all solutions were filtered with 0.22μm filter units to remove particulate matter.

4.4.2 Results

Figure 4.13 shows the distribution of gold particles along the D-period of collagen fibrils formed *in vitro* in the presence and absence of TRAMP. In the presence of TRAMP, the majority of gold particles were found between the b_1 and e_2 bands (Figure 1.1). However, peaks were also observed in this area when fibrils were formed in the absence of TRAMP and also when the TRAMP antibody was replaced with pre-immune serum or buffer alone. This suggests that the preference of the gold probe for the area between the b_1 and e_2 bands is due to non-specific charge attractions and not binding to TRAMP within the fibril.

Attempts were made to reduce the non-specific binding. The blocking step was modified so that 10% normal goat serum (Scottish Antibody Production Unit) was used in place of PBST and the incubation times were increased to 1 hour. Affinity purified TRAMP antibody (section 2.7) was used and the primary antibody incubation time was increased to 16 hours at 4°C. Dilutions of primary antibody were carried out with 0.7mM Na_2HPO_4, 0.3mM KH_2PO_4, 150mM NaCl, 0.1% BSA, 0.1% Tween-20, pH 7.2. Between primary and secondary antibody incubations the grids were washed thoroughly on 3 x 50μl droplets of Tris I (0.05M Tris-HCl, pH 7.4) for 1 min each, followed by 3 x 50μl droplets of Tris II (0.05M Tris-HCl, 0.1% BSA, pH 7.4) for 1 min each and finally on a 50μl droplet of Tris III (0.05M Tris-HCl, 1% BSA, pH 8.2) for 15 mins. Tris is believed to stabilise bound gold particles (Miss Kate Gregory, oral communication, August 1996). The second antibody incubation step was unchanged, after which grids were washed on a series of 50μl droplets for 3 x 1 min on Tris II, 3 x 1 min on Tris I and 3 x 1 min on ddH_2O. The washing steps were further improved by constant agitation achieved by placing grids on a drop of liquid on top of a magnetic stirrer which allows them to rotate.
Figures 4.14 and 4.15 show the results of these modifications. Background levels of binding of the gold probe to collagen fibrils in the absence of TRAMP antibody were

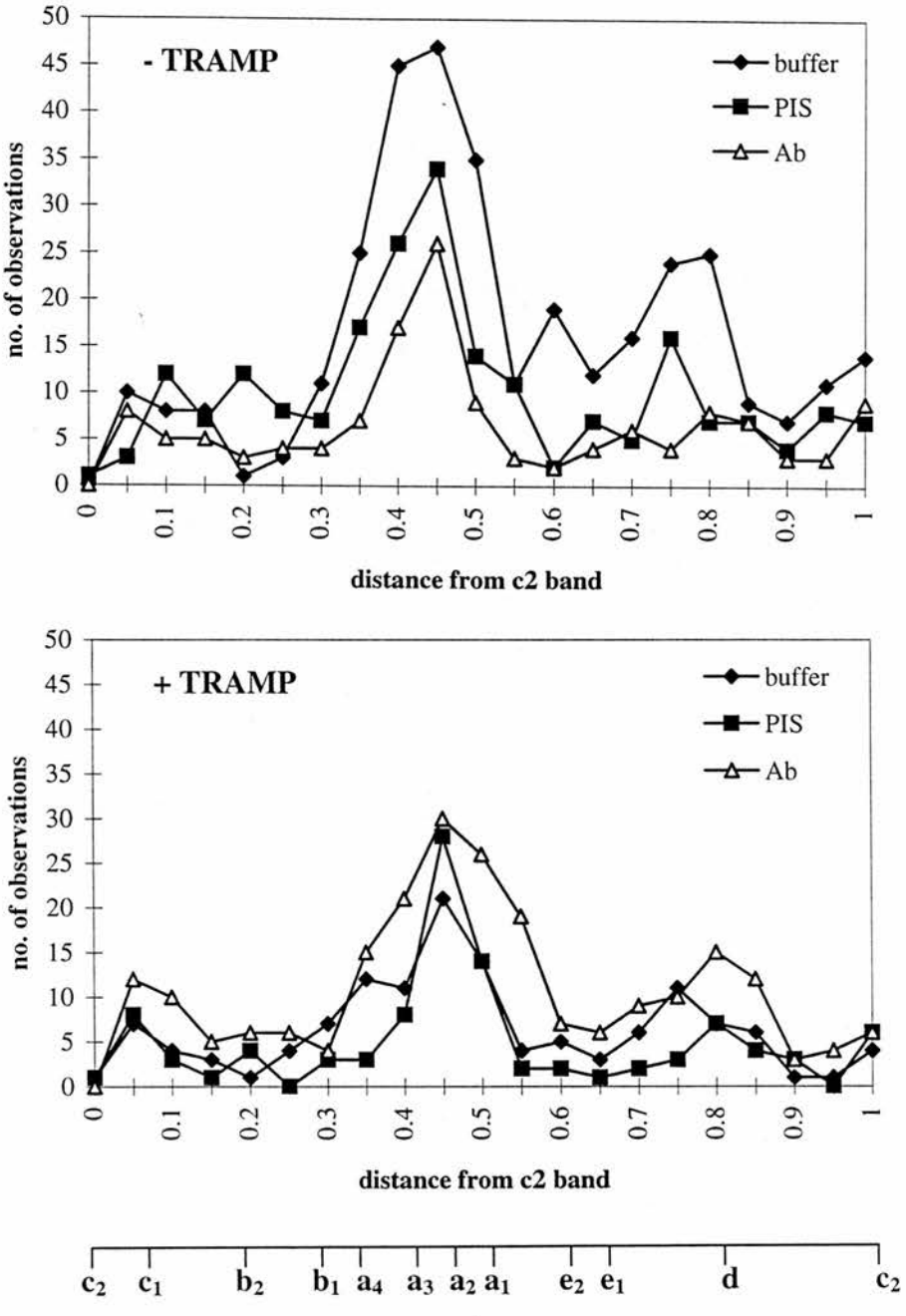


Figure 4.13. Immunogold localisation of TRAMP on *in vitro* reconstituted collagen I fibrils using polyclonal antiserum to TRAMP. Graph shows distribution of gold particles along the D-period from the 'c_2' band in the absence and presence of TRAMP. Primary antibody (Ab) was a 1:1000 dilution of polyclonal antiserum to TRAMP. PIS represents a 1:1000 dilution of pre-immune serum.

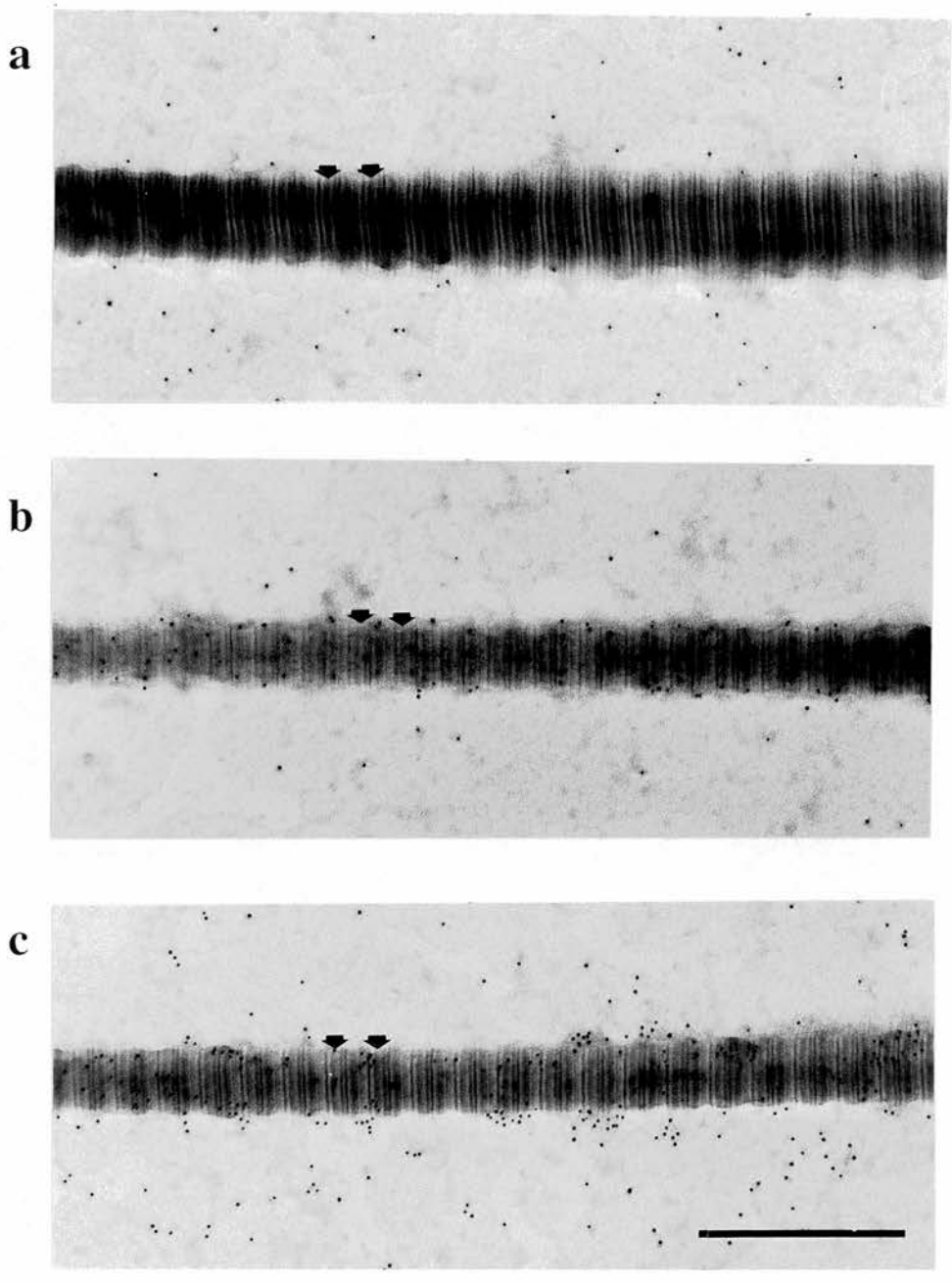


Figure 4.14. Typical electron micrographs after immunogold labelling of collagen I fibrils reconstituted *in vitro* in the absence (a, b) or presence of TRAMP (c). In (a) grids were incubated with diluting buffer in place of primary antibody whilst in (b) and (c) the primary antibody was undiluted affinity purified antiserum to TRAMP. Arrows denote the location of the c_2 band. The scale bar represents 300nm.

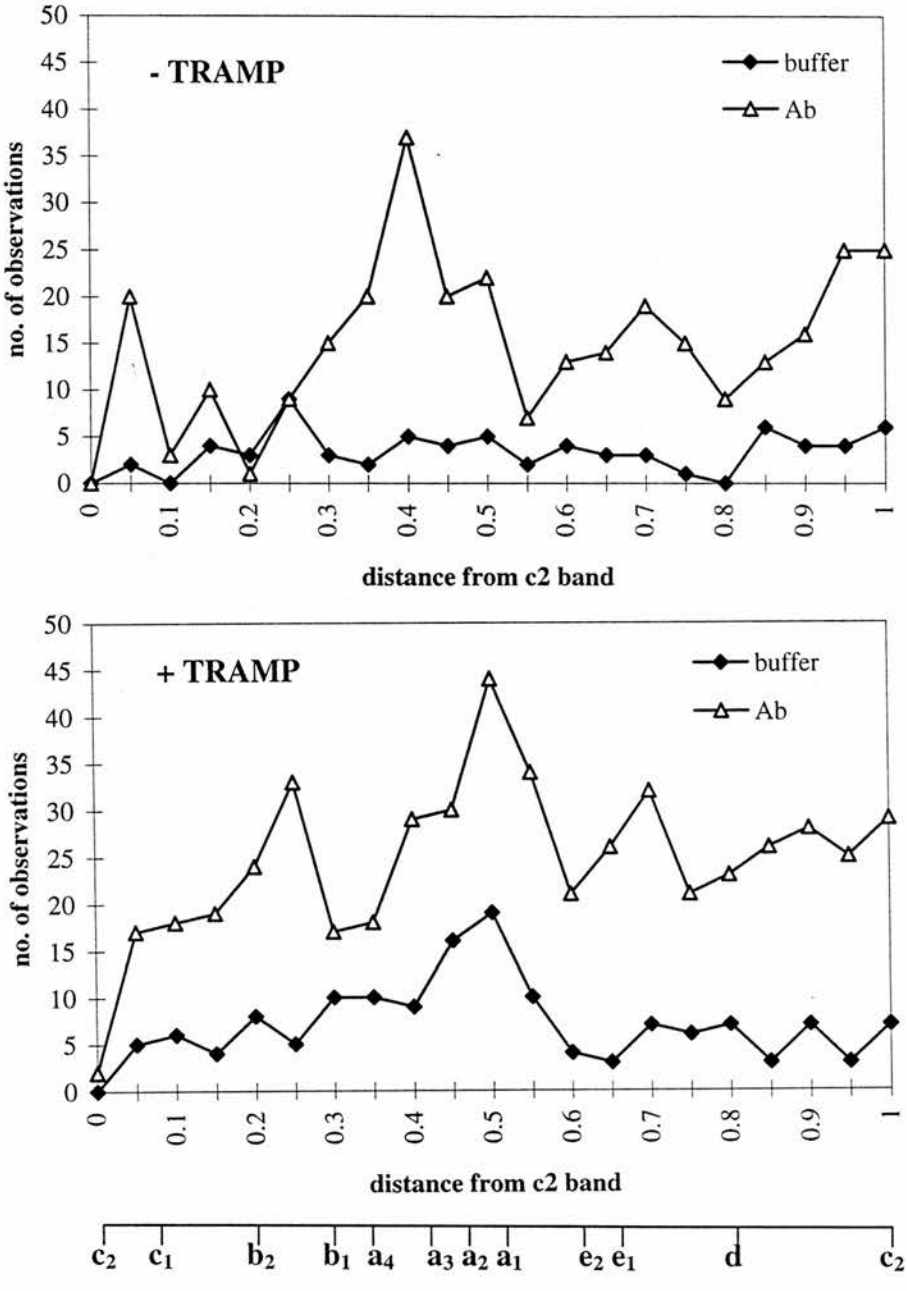


Figure 4.15. Immunogold localisation of TRAMP on *in vitro* reconstituted collagen I fibrils using affinity purified antiserum to TRAMP. Graph shows distribution of gold particles along the D-period from the 'c_2' band in the absence and presence of TRAMP. Primary antibody (Ab) was undiluted affinity purified antiserum to TRAMP (section 2.7).

decreased both in the presence and absence of TRAMP, although a small peak was still observed between the b_1 and e_2 bands in the presence of TRAMP. However, the modifications were unsuccessful in decreasing non-specific binding in the presence of primary antibody since a peak of gold particles was again observed between the b_1 and e_2 bands both in the presence and absence of TRAMP. An overall increase in the number of gold particles bound to fibrils was observed in the presence of TRAMP and higher distributions were found between the b_2 and b_1 bands and the a_2 and e_2 bands. However, the significance of these peaks is unclear since they are close to the non-specific binding peak and depend on the accuracy of the counting procedure. These results suggest that there are no specific areas of binding of TRAMP to collagen fibrils or that non-specific binding is still masking these areas.

4.5 Discussion

The results of this study confirm that reconstituted collagen fibrils similar to fibrils formed *in vivo* can be reproducibly obtained from purified lathyritic rat skin collagen I. Also, lowering the concentrations of phosphate and/or TES increase the rate of fibril assembly as judged by the change in turbidity over time. The presence of phosphate and TES ions is known to inhibit fibrillogenesis probably due to their association with free collagen molecules and/or the growing fibril, thereby disrupting collagen-collagen interactions (Holmes *et al.*, 1986; Pogany *et al.*, 1994). An alternative hypothesis suggests that the effect of these anions on fibril assembly is indirect and due to their ability to increase the order of bulk water, thus making fibrillogenesis energetically less favourable (Cooper, 1970). It should be noted that the complete inhibition of fibril formation at 100µg/ml collagen in 30mM TES, 30mM Na_2HPO_4, 135mM NaCl, pH 7.4 contradicts previous reports by Macbeath *et al.* (1993). This may be explained by the observation that the kinetics of assembly for a particular collagen sample are sensitive to the exact method used to purify it. The present study also shows that TRAMP purified by DEAE-Sephacel anion exchange followed by Superdex-75 gel filtration and Mono Q FPLC (chapter 3) is still able to accelerate fibril assembly. Decreasing the phosphate and TES concentration has no

affect on the ability of TRAMP to accelerate fibrillogenesis, suggesting that TRAMP would be capable of eliciting this effect under physiological conditions.

The amount of TRAMP associated with collagen fibrils is reduced when the concentration of phosphate in the buffer is decreased. This may be the result of the speed at which fibrils are formed at low phosphate concentrations since there would be less time for TRAMP to bind collagen before fibrillogenesis began. The fact that removing TES from the buffer had no further affect on the binding of TRAMP to collagen fibrils suggests that the interaction is not via the sulphate groups on TRAMP since if this was the case, both phosphate and the sulphonic acid group on TES would be expected to compete for binding with TRAMP. Thus binding would be more efficient at low phosphate and TES concentrations.

A number of possible explanations exist for the lack of binding of TRAMP to pre-formed collagen fibrils. Firstly, any interference with the system during fibrillogenesis is known to affect the kinetics and morphology of fibrils. Thus, layering may disrupt the interaction of TRAMP with fibrils. Secondly, the observations may be genuine, suggesting that the TRAMP-collagen fibril interaction ceases after long incubation periods. Lastly, the lack of binding fibrils formed in the presence of TRAMP may be the result of the speed at which the fibrils formed. At such high starting concentrations of collagen and using the 'warm start' technique, it is reasonable to expect fibrillogenesis to be essentially instantaneous with no measurable lag phase. If this is the case, TRAMP may not be able to bind to collagen before fibrillogenesis commences and therefore cannot be incorporated into fibrils. The fact that TRAMP is still bound to collagen fibrils after 40 hours at 34°C if no buffer is added suggests that the first of these possibilities is the most likely.

Partial removal of sulphate groups had no affect on the ability of TRAMP to accelerate fibril formation, suggesting that tyrosine sulphation is not important for this function of the protein. However, the possibility that any detrimental effect can only be observed after complete removal of sulphate groups cannot be ruled out. Although the role of tyrosine sulphation in proteins remains unclear it has been suggested that it may be involved in regulating the various activities of

multi-functional proteins. For example, partial desulphation of fibronectin has no affect on its gelatin and heparin binding activity but results in a decrease in its ability to bind fibrin (Suiko and Liu, 1988; section 1.6.2). Thus, tyrosine sulphation may be important for an as yet unidentified function of TRAMP.

It appears that the binding affinity of TRAMP for collagen I monomers is much higher than for fibrils. Taking 22kDa and 300kDa as the respective molecular masses of TRAMP and collagen, and assuming that all TRAMP added to the well binds up to saturation and that 1μg of collagen is bound per well, the highest concentration of TRAMP (i.e. 10μg/ml) gave a molar ratio of TRAMP to collagen of 27 to 1. This compares with a 1:2 ratio at saturation for TRAMP binding to collagen fibrils (Macbeath *et al.*, 1993). The higher affinity of TRAMP for collagen monomers compared to fibrils supports the theory that this protein is involved in the early nucleation phase of fibrillogenesis. Macbeath *et al.* (1993) suggested that TRAMP facilitated and/or stabilised the assembly of 4D-staggered dimers during the nucleation phase (section 1.7.2). Assuming that the immobilised collagen is solely monomeric, has similar binding properties to free collagen and that there is no self-aggregation of TRAMP, this ratio of TRAMP to collagen monomers would mean that collagen monomers would be covered with a layer of TRAMP molecules. However, the low levels of TRAMP found in skin compared to collagen suggest that this is unlikely to occur *in vivo* (section 4.1; Chapter 3). The lower affinity of TRAMP for collagen fibrils may also help to explain the results of the immunogold labelling of the collagen fibril. If the binding of TRAMP to the fibril is relatively weak it will be easily displaced by washing steps.

TRAMP is unable to reverse the inhibitory effect of decorin on fibril formation thus providing evidence that these two ECM components may work together to regulate the extent of fibril formation. How decorin exerts this effect is unclear. It does not appear to be the result of displacement of TRAMP from fibrils since the presence of decorin enhances TRAMP binding to collagen fibrils in TES buffer. Decorin is also unable to displace TRAMP from collagen monomers or enhance its binding in the solid phase assay. However, interpretation of the results of the co-sedimentation and solid phase assays are complicated by the fact that decorin and TRAMP not only bind

to collagen but also to each other (Okatamo *et al.,* 1996). Thus, the apparent enhancement of binding to collagen fibrils may be due to TRAMP binding to collagen via decorin in addition to binding to collagen directly. The fact that no TRAMP is pelleted after incubation with decorin alone suggests that the increase is not an artefact caused by precipitation of aggregates of TRAMP and decorin. It is also of interest to note that in 10mM PBS the presence of decorin has no affect on binding of TRAMP. Again, this may be the result of the speed of fibril formation at low phosphate concentrations preventing interactions between TRAMP, decorin and collagen.

Both the solid phase and co-sedimentation assays suggest separate binding sites for TRAMP and decorin on collagen since no competition was observed. However, this could not be confirmed by immunogold labelling of collagen fibrils. Decorin has been localised to the d and e_2 bands on positively stained collagen fibrils *in vivo* using monoclonal antibodies (Scott and Orford, 1981; Pringle and Dodd, 1990). By comparison the present study shows no obvious preferred binding sites for TRAMP on the fibril although a study using monoclonal antibodies would be necessary to confirm this. Another possible explanation for the lack of results by immunogold labelling is that the TRAMP is localised in the interior of the fibril and thus its antigenic sites are not available for antibody binding. Decorin was shown by electron microscopy followed by rotary shadowing to have 2 binding sites on collagen I monomers approximately 50 and 100nm from the N-terminal end of the molecule (Yu *et al.*, 1993). Similar experiments with TRAMP would be useful to confirm the separate binding sites of TRAMP and decorin. It should also be noted that at saturation the levels of binding of decorin to collagen are much lower than for TRAMP. Hedbom and Heinegard (1989) showed saturation at molar concentrations of decorin 3 to 4 times that of collagen whilst the highest molar concentration of TRAMP used in the present study was 27 times greater than that of collagen. It is therefore possible that collagen has more than 2 binding sites for TRAMP.

Both decorin and TRAMP decrease the diameters of collagen fibrils (Vogel and Trotter, 1987; Macbeath *et al.,* 1993; section 1.7). In the case of decorin, this is postulated to be the result of inhibition of the lateral association of collagen

monomers whereas the end-to-end association of monomers with oligomers and microfibrils is unaffected (Rosenberg *et al.,* 1993; Danielson *et al.*, 1997). Three hypotheses have been put forward to explain how TRAMP produces thinner fibrils. Firstly, it may increase the number of nucleation sites. Secondly, it may alter the nature of the nuclei so that the axial (i.e. end-to-end) growth is favoured over lateral growth. Finally, it was suggested that TRAMP may form a circumferential layer around the fibril once a 'critical' diameter is reached so that further addition of monomers is blocked (Macbeath *et al.,* 1993). The low levels of TRAMP localised on collagen fibrils by immunolabelling refutes the last of these hypotheses. If decorin and TRAMP act on different parts of the fibril assembly mechanism, it is reasonable to expect them to bind at different sites on the collagen molecule. It also suggests that *in vivo* their combined role is not only to regulate the rate of fibril assembly but also the morphology of the fibrils formed. In addition, this has implications for the differences in morphology of collagen fibrils from different tissues.

Chapter 5

Immunological and Enzymatic Studies on TRAMP

5.1 Immunological studies

Tyrosine rich acidic matrix protein (TRAMP) has previously been shown by western blot analysis with polyclonal anti-serum to have a widespread tissue distribution in both murine and porcine tissues (Forbes *et al.*, 1994; section 1.6.3). The aims of the present study were to look at a wider range of murine tissues using both western blotting and immunohistochemistry methods.

5.1.1 Immunohistochemistry

5.1.1.1 Methods

Immunohistochemistry procedures described below were carried out by Amanda Robson, Roslin Institute, Edinburgh. Tissues were removed from freshly killed 21 day old C57 black mice and frozen immediately, either by immersion in isopentane, previously cooled with solid CO_2, or by suspension above liquid nitrogen, followed by storage at -80°C. For cryo-microtomy, specimens were attached to chucks with OCT compound (BDH/Merck) and 10µm sections were cut at -30°C using a Shandon model OT cryostat. Sections were then transferred to poly-L-lysine coated slides and endogenous peroxidase activity was blocked with 0.6% (v/v) hydrogen peroxide in methanol. Slides were indirectly immunolabelled with undiluted affinity purified antibodies to TRAMP (section 2.7 and 4.2.5) for 2 hours at 4°C, followed by biotinylated secondary antibody (Vectastain Elite ABC kit). The slides were then stained with 3,3'-diaminobenzidine (DAB) and 0.09% hydrogen peroxide followed by light counterstaining with haemotoxylin. Coverslips were placed over the slides which were mounted with DPX (BDH/Merck). In control slides primary antibody was replaced either with an irrelevant antibody or with PBS. Additionally, paraffin sections stained with haemotoxylin and eosin were prepared for use as a reference to orientate cryo-sections.

5.1.1.2 Results

Cryo-sections from mouse skin showed diffuse staining for TRAMP throughout the dermis (Figure 5.1a,b). Diffuse staining was also observed within connective tissues surrounding the muscle fibres in skeletal muscle sections (Figure 5.1c,d) and in the endocardium, myocardium, blood vessel walls and supportive tissue of the heart (Fgure 5.1h) Strong staining was observed in the serosa and lamina propria of the duodenum (Figure 5.1e,f) and in the sclera of the eye (Figure 5.1g). In addition, TRAMP was localised to the wall of the corpus lutia of the ovary and also within the kidney (data not shown). No TRAMP was identified in cryo-sections from the testes and liver (data not shown).

5.1.2 Western blot analysis of murine tissues

5.1.2.1 Methods

Prior to western blot analysis (section 2.4), tissues that had been rapidly frozen for cryo-microtomy were extracted in 50mM Tris-HCl, 0.15M NaCl, pH 7.5 containing 2% (w/v) SDS and 1mM PMSF for 10 minutes at 100°C according to the method of Kessler *et al.* (1990). For western blotting a 1:5000 dilution of the anti-TRAMP antibody was used.

5.1.2.1 Results

Figure 5.2 shows TRAMP to be present in large amounts in murine bladder, ovary and tail with smaller amounts detectable in testes, skeletal muscle, lung and duodenum. No TRAMP was detected in extracts from brain, spleen or eye.

5.2 Enzymatic studies

The possible amine oxidase activity of TRAMP (section 1.6.4.3) was investigated using benzylamine, elastin and collagen substrates. Additionally, attempts were made to identify the presence of quinones within the TRAMP sequence.

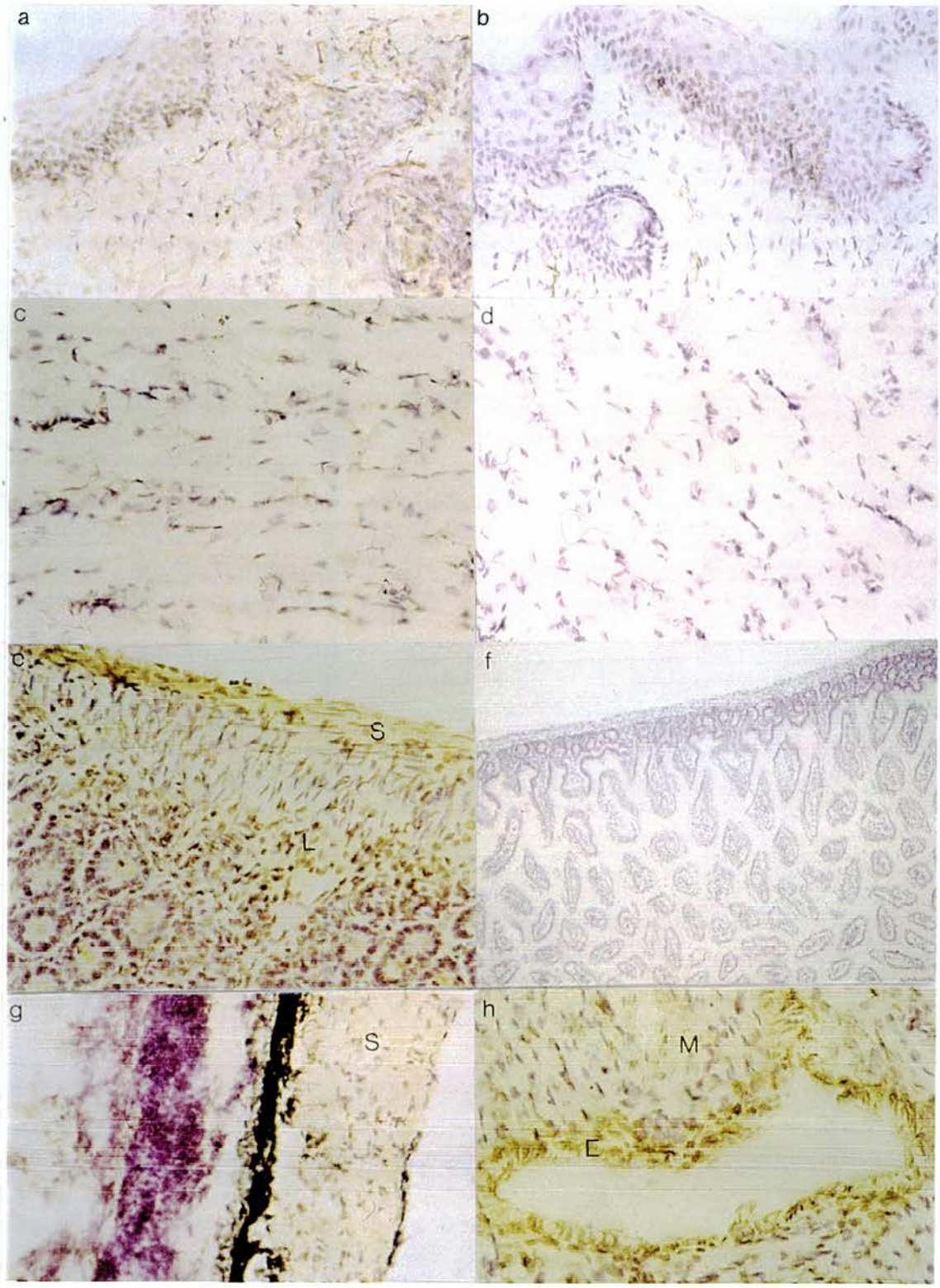


Figure 5.1. Localisation of TRAMP in mouse tissues by immunohistochemistry. (a,b) skin, 1460x. (c,d) skeletal muscle, 1460x. (e,f) duodenum. The serosa (S) and lamina propria (L) are labelled, (e) 1460x, (f) 365x. (g) eye, S represents sclera, 1460x. (h) heart, The endocardium (E) and myocardium (M) are labelled, 1460x. (a,c,e,g,h) Neat affinity purified anti-TRAMP antibodies. (b,d,f) Controls.

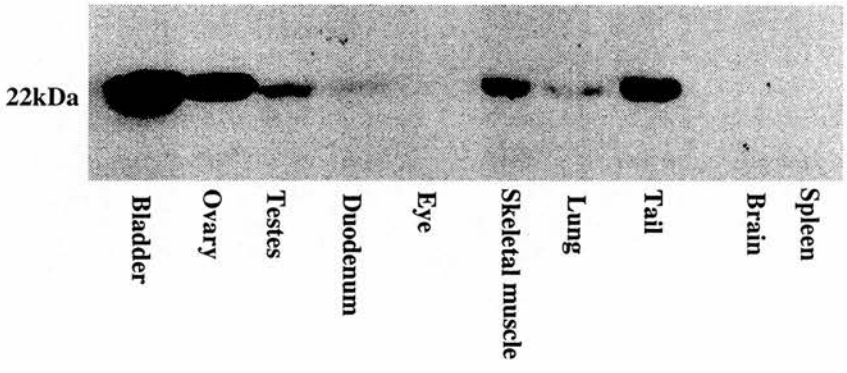


Figure 5.2. Detection of TRAMP in murine tissue extracts by SDS-PAGE and western blotting. 100μg protein was loaded in each lane. Electrophoresis was carried out in reducing conditions with 12% acrylamide in the separating gel. Antibody detection (1:5000 dilution) was by enhanced chemiluminescence.

5.2.1 Assays for amine oxidase activity

5.2.1.1 Benzylamine assay

Detection of amine oxidase activity on a benzylamine substrate was carried out as described by Tabor *et al.* (1954) in collaboration with Dr. Euan Forbes. Briefly, 20μg/ml of TRAMP was incubated with 1mM benzylamine hydrochloride in 0.1M sodium phosphate buffer, pH 7.2 in a total volume of 1ml and production of benzylaldehyde was monitored at 250nm at 25°C in a Phillips PU 8720 UV/VIS spectrophotometer. Bovine serum amine oxidase (BSAO; Sigma) previously purified by affinity chromatography on EAH Sepharose 4B (Pharmacia) according to the method of Houen *et al.* (1993), was used as a positive control. Partially purified recombinant human lysyl oxidase (kindly donated by Dr. M. Ouzzine; Ouzzine *et al.*, 1996) was also assayed. Controls were included with the addition of 0.2mM β-APN.

5.2.1.2 Lysyl oxidase assay with elastin substrate.

For detection of amine oxidase activity of TRAMP on an elastin substrate, the ultrafiltration assay described in section 2.1 was used. In general, 2μg of either TRAMP or a mixture of lysyl oxidase and TRAMP were used in each assay and controls were included with the addition of 0.2mM β-APN.

5.2.1.3 Lysyl oxidase assay using a collagen substrate

For detection of amine oxidase activity of TRAMP on a collagen substrate a modification of the lysyl oxidase ultrafiltration assay (section 2.1) was used. Chick skin was used as the source of collagen in preference to chick calvaria since lysine residues in the telopeptide of skin collagen are unhydroxylated (Seyedin and Rosen, 1990). Thus, tritium would not be lost from the 5 position in [4,5 -^{3}H] lysine labelled collagen during hydroxylation and would be available for assay of lysyl oxidase/amine oxidase activity (see Figure 1.2).

5.2.1.3.1 Preparation of [4,5-^{3}H] lysine labelled collagen from chick skin

The method used for preparation of [4,5-^{3}H] lysine labelled collagen was based on the methods of Siegel (1974) and Payne *et al.* (1986). The skins from 10 dozen, 17 day old chick embryos (~15g wet weight) were removed and labelled with [4,5-^{3}H] lysine as described for aortas in section 2.1.1. The skins were washed in Hanks balanced salt solution (HBSS), chopped up manually using a scalpel and then homogenised in a glass-teflon homogeniser with ~ 50ml of 50mM Tris-HCl, 1M NaCl, pH 7.5 containing 20mM EDTA, 10mM NEM and 1mM PMSF. The homogenate was diluted to 200ml with the same buffer and extracted overnight at 4°C with end over end rotation. After centrifugation at 28,000*g* for 45 minutes the supernatant was collected and subjected to salt precipitation (to remove collagen III) with addition of NaCl up to a final concentration of 2M. The precipitate was allowed to form for 5 hours and again the extract was centrifuged at 28,000*g* for 45 minutes. The supernatant was collected and NaCl was added up to a final concentration of 3.5M and left to precipitate overnight. The centrifugation step was repeated and the pellet was washed in 3.5M NaCl, 50mM Tris-HCl, pH 7.5 containing 20mM EDTA, 10mM NEM and 1mM PMSF, before redissolving in 200ml DEAE-start buffer (0.2M NaCl, 50mM Tris-HCl, pH 7.5) overnight. The sample was dialysed against two changes of this buffer and centrifuged at 10,000*g* for 45 minutes before loading onto a DEAE-Sephacel column (2.6 x 20cm) at a flow rate of 20ml/hour. Fractions containing ^{3}H were pooled (525ml), concentrated to 45ml and dialysed against 2 changes of PBS, pH 7.8.

5.2.1.3.2 Assay for amine oxidase activity on collagen substrate

A modification of the ultrafiltration assay described in section 2.1.2.1 was used to assay for amine oxidase activity of TRAMP on a collagen substrate. In initial experiments, 100µl of ^{3}H collagen was diluted with 700µl of PBS and incubated with 100µl of TRAMP (3µg) or PBS at 37°C for 20 hours. However, fibrillogenesis assays of the assay mixture showed no fibril formation after 4 hours probably due to the relatively low concentration (156µg/ml) of collagen present in the final assay

mixture. Thus, to ensure that fibril formation occurred the assay mixture contained 800µl of collagen substrate and 100µl of TRAMP or PBS, giving a final collagen concentration of 1.25mg/ml. Controls containing 0.2mM β-APN and buffer alone were also included.

5.2.2 Results

Table 5.1 summarises the results of amine oxidase assays of TRAMP and lysyl oxidase on benzylamine, elastin and collagen substrates. Whilst amine oxidase activity was observed for BSAO in the benzylamine assay and for lysyl oxidase in the elastin assay after subtraction of β-APN blanks, TRAMP showed no amine oxidase activity with either of these substrates. When collagen was used as a substrate, TRAMP appeared to have some activity after subtraction of the β-APN blank (450cpm/ml). However, this value is lower than the background levels obtained for buffer alone (1390cpm/ml) and thus TRAMP had no measurable amine oxidase activity on the collagen substrate.

5.3 Detection of quinoproteins by redox-cycling staining

5.3.1 Methods

The procedures described below were carried out by Dr. Euan Forbes.

The method of Paz *et al.* (1991) was used for detection of quinoproteins by redox-cycling staining. This method is based on the ability of quinones to oxidise glycine to form a superoxide which then reduces nitroblue tetrazolium to its formazan giving rise to a dark blue colour. BSAO previously purified by affinity chromatography on EAH Sepharose 4B (Pharmacia) according to the method of Houen *et al.* (1993) was used as a positive control. TRAMP (100µg) and BSAO (100µg) were subjected to SDS-PAGE (section 2.3) and transferred to nitrocellulose by electroblotting (section 2.4.1.2). Blots were incubated in 2M potassium glycinate/0.24M nitroblue tetrazolium, pH 10 for 45 minutes in the dark.

	Benzylamine (μM converted/sec)		Elastin (cpm/ml)		Collagen (cpm/ml)	
	- β-APN	+β-APN	- β-APN	+β-APN	- β-APN	+β-APN
buffer alone	0	0	9490	8760	4780	3390
BSAO	6.5	0	nd	nd	nd	nd
lysyl oxidase	0	0	13420	9700	nd	nd
TRAMP	0	0	10680	10810	3740	3290

Table 5.1 Summary of results of assays for amine oxidase activity of TRAMP and lysyl oxidase on various substrates. It should be noted that in the benzylamine assay recombinant lysyl oxidase was assayed whilst in the elastin assay a mixture of lysyl oxidase and TRAMP was assayed. nd = not determined.

5.3.2 Results

Figure 5.5a shows staining of BSAO but not TRAMP by this method suggesting that TRAMP is not a quinoprotein.

5.4 Discussion

Previously, TRAMP has been shown by western blotting to be present in murine skin, lung, cartilage, bone, skeletal muscle, heart and kidney (Forbes *et al.*, 1994; section 1.6.3). In the present study TRAMP was also found to be present in duodenum, testes, ovary and bladder. Affinity purification of the antibody prior to immunohistochemistry successfully eliminated the problems of non-specific binding, (Robson, oral communication) and allowed further localisation of TRAMP within these tissues to the extracellular matrix. The present study thus confirms that TRAMP is widespread in connective tissues. In general, there was good agreement between the results obtained by western blotting and immunohistochemistry with some exceptions. Firstly, although a positive signal was obtained by western blotting, no staining was observed within testes and bladder sections by immunohistochemistry. This may be explained either by the enhanced sensitivity of the western blotting procedure or by the fact that anti-TRAMP antibodies which had not been affinity purified were used for western blotting. More difficult to explain is the strong staining within the sclera of the eye observed by immunocytochemistry and the negative result for eye extracts by western blotting. It is possible that the antibody cross-reacts with a low molecular weight species not present after SDS-PAGE with 12% acrylamide in the separating gel. The localisation of TRAMP to the dermis in skin is in agreement with previous findings for the human equivalent of TRAMP, dermatopontin (Okamato *et al.*, 1996).

The data presented here suggests that TRAMP does not contain a quinone cofactor and has no amine oxidase activity. Thus, the ability of TRAMP to stabilise collagen fibrils against low temperature dissociation (section 1.6.4.2; Macbeath *et al.*, 1993) is

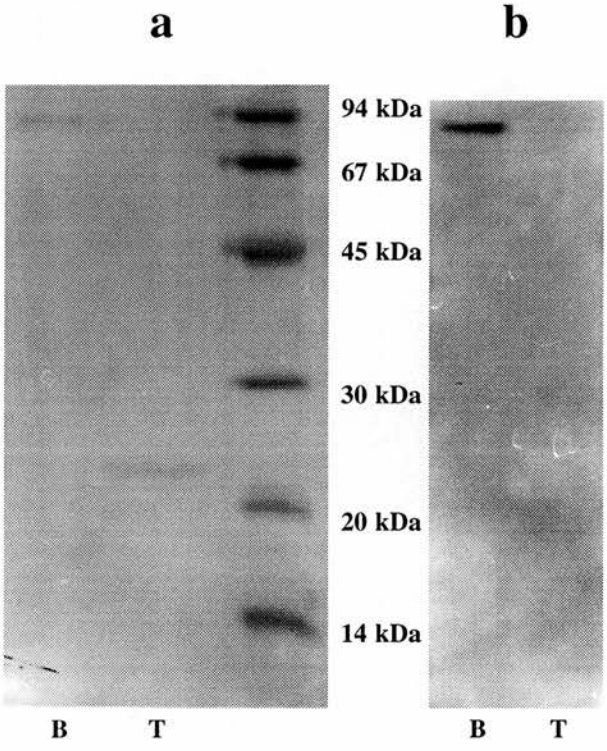


Figure 5.3. Detection of quinoproteins by redox cycling staining. TRAMP (T; 22 kDa) and bovine serum amine oxidase (B; 90kDa) were subjected to SDS-PAGE with 12% acrylamide in the separating gel and stained with Coomassie blue (a) or blotted onto nitrocellulose and stained by redox cycling with 2M potassium glycinate, 0.24mM nitroblue tetrazolium, pH 10.

unlikely to be the result of the lysyl oxidase-like activity. The lack of amine oxidase activity with the collagen substrate contradicts previous, unpublished observations by Forbes (1994) which suggested that the presence of TRAMP led to the formation of intramolecular crosslinks (section 1.6.4.3). This may be explained either by the fact that a different source of collagen was used in the present study or that the TRAMP used was of higher purity and thus free from contaminating lysyl oxidase. However, the possibility that this method is not sensitive enough to detect the low levels of conversion of lysine to allysine cannot be ruled out. Since TRAMP has no detectable amine oxidase activity and is not a quinoprotein, the presence of the consensus NYD sequence found in TRAMP, 22K ECM protein and dermatopontin appears to have no functional significance.

Chapter 6

Final Discussion

In the present study TRAMP, free from lysyl oxidase, was successfully purified from porcine skin although yields remained low. If the denaturing effect of guanidine hydrochloride on TRAMP is reversible, it may be possible to further improve the yields of TRAMP by using 4M guanidine hydrochloride in place of urea during the initial tissue extraction (Okamato *et al.*, 1996). The use of FPLC/HPLC columns throughout the purification may also help to improve resolution and decrease the time scale of the purification process. Alternatively, increased yields of TRAMP may be obtained by development of a bacterial or eukaryotic expression system. Such a system would also eliminate many of the problems encountered during conventional purification due to the presence of lysyl oxidase.

The physiological significance of the close association between TRAMP and lysyl oxidase remains unclear. It would therefore be of interest to examine the interactions between these two proteins in more detail. This could be achieved by competitive ELISAs or by density gradient centrifugation. Additional information as to whether TRAMP and lysyl oxidase interact *in vivo* may be provided by double immunolabelling of tissue sections at the electron microscopy level to directly compare their distributions. However, due to the apparent cross-reactivity of the lysyl oxidase antibody and the low avidity of the TRAMP antibody this would require the production of highly specific monoclonal antibodies.

TRAMP has previously been shown to bind collagen I fibrils and accelerate collagen I fibril formation *in vitro* (Macbeath *et al.*, 1993). In this study, TRAMP was also shown to bind collagen I monomers with higher affinity than to fibrils and a role in the early stages of fibril assembly has been suggested. Future experiments on the interactions between TRAMP and collagen I monomers should focus on their binding in solution. This could be achieved using a modification of the solid phase assay used here (section 4.2.2.2.2) with competition for TRAMP binding between collagen immobilised on wells and free in solution (Hedbom and Heinegard, 1993). This would also allow more accurate analysis of binding saturation and the number of binding sites for TRAMP on collagen. The binding sites of TRAMP on collagen I monomers could be further investigated by rotary shadowing and electron

microscopy. Since TRAMP is a relatively small protein it is at the limit of resolution of this technique and thus visualisation would probably entail the use of an anti-TRAMP antibody. Additionally, it will be of interest to compare the binding affinities of other fibrillar and non-fibrillar collagens for TRAMP and to investigate possible roles of TRAMP in their assembly.

Partial removal of the sulphate groups from TRAMP had no affect on its ability to accelerate fibril formation *in vitro*. Future work should focus on the effect of partial removal of sulphate groups on binding to collagen I monomers and fibrils and to decorin. Additionally, complete removal of sulphate groups will be necessary to elucidate more fully whether they have a role in accelerating the fibrillogenesis process.

Another interesting set of experiments would involve the production of a number of TRAMP peptides either synthetically, by enzymatic cleavage or using recombinant methods. These peptides could then be studied in the fibrillogenesis and binding assays to identify sequences important for the acceleratory effect of TRAMP on fibril formation and/or for binding to collagen fibrils and monomers.

Although TRAMP is found associated with collagen fibrils *in vitro* and has been localised to the extracellular matrix of murine tissues by immunohistochemical methods, there is no direct evidence to date that TRAMP is localised on or within collagen I fibrils *in vivo*. It will therefore be valuable to use immunogold labelling to localise TRAMP in tissues at the electron microscopy level. As mentioned earlier this would require the production of highly specific, monoclonal antibodies to TRAMP to overcome the problems encountered during immunogold labelling of *in vitro* reconstituted collagen fibrils. It will also be important to check that the inability to identify specific binding sites for TRAMP on collagen fibrils formed *in vitro* is not due to masking of antigenic sites by closely packed collagen molecules, as is the case with heterotypic fibrils of collagens I and V (Birk *et al.*, 1988). To do this the fibrils should be treated with acetic acid or collagenase to disrupt the fibril structure prior to commencing the labelling procedure.

The results of the present study suggest that the interactions between TRAMP, decorin and collagen I are complex. They are also of particular interest given the profound effect of decorin gene "knockouts" on the supramolecular architecture and spatial arrangement of collagen fibrils within the skin (Danielson *et al.*, 1997). Double immunogold labelling of TRAMP and decorin at the electron microscope level may give further insight into the interplay between these ECM components. Additionally, identification of the binding site(s) of TRAMP on decorin, with particular reference to the three-dimensional shape of this SRLP and its proposed modes of interaction with collagen I, may provide important pointers as to the role of TRAMP in fibril assembly. Likewise, comparisons of the binding sites of decorin and TRAMP on collagen I monomers and fibrils and elucidation of the binding sites of decorin on TRAMP will help to complete the picture. It may even be possible to study the decorin-TRAMP-collagen complex by rotary shadowing. The potential interactions of TRAMP with other members of the SRLP family could also be investigated.

The results of western blotting and immunohistochemical studies presented here confirm previous reports that TRAMP is a widely distributed connective tissue constituent. In the future, further understanding of the role(s) of TRAMP in the ECM may also be gained by studying the developmental localisation and expression of TRAMP in murine tissues using the above methods and also by Northern blotting or *in situ* hybridisation. Additional studies on the distribution and expression levels of TRAMP in tissues from patients with hereditary connective tissue disorders may serve to implicate TRAMP gene mutations in these diseases.

One possible function of TRAMP which was not addressed in the current work is its role in the attachment of cells to the ECM. Possible cell surface receptors for TRAMP could thus be identified, by subjecting detergent cell extracts to chromatography on a TRAMP affinity column. An alternative and perhaps more labour intensive approach would be to use antibodies to the various classes of integrins in order to block cell adhesion of TRAMP to mouse 3T3 cells or human dermal fibroblasts.

In the future, a more effective way to study the function(s) of TRAMP in the extracellular matrix may be to look at the supramolecular architecture of the matrix after TRAMP gene disruption or knockout experiments.

Appendix

Localisation of procollagen C-proteinase enhancer in murine tissues

A1. Introduction

The procollagen C-proteinase enhancer (PCPE) is a 55kDa glycoprotein which enhances the activity of procollagen C-proteinase/bone morphogenetic protein-1 (BMP-1; Kessler and Adar, 1989; section 1.2.2). Both 34kDa and 36kDa fragments of PCPE have also been identified although their physiological significance remains to be elucidated (Adar *et al.*, 1986). PCPE has previously been shown by western blotting and RNase protection to be widespread within connective tissues (Kessler *et al.*, 1990; Takahara *et al.*, 1994). The aims of the current study were to test a number of other murine tissues for the presence of PCPE by western blotting. The results of western blots could then be compared to concurrent investigations by immunocytochemical localisation carried out by Amanda Robson.

A2. Western blotting

Tissues which had been removed from 21 day old C57 black mice and rapidly frozen for cryo-microtomy were extracted as described in section 5.1.2.1 and subjected to SDS-PAGE and western blotting (section 2.4). The primary antibody (obtained from Dr. E. Kessler) had previously been raised against the 36kDa fragment of PCPE and affinity purified as described in section 2.7. This antibody was used at a 1:500 dilution.

The results confirm previous reports of the presence of PCPE in skeletal muscle and eye. In addition, the present study showed PCPE to be present in lung, testes, ovary and bladder (Figure A1).

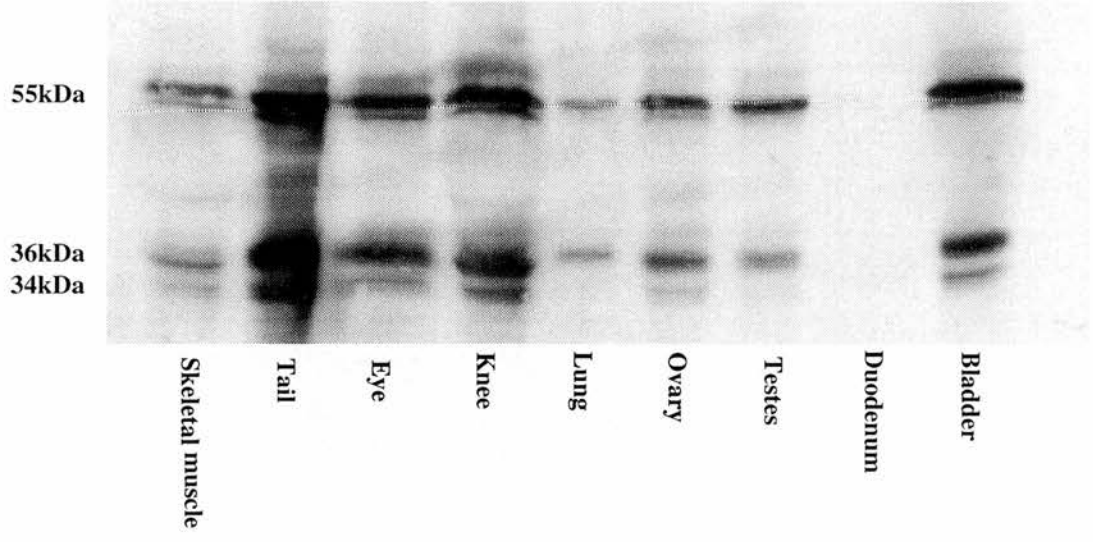


Figure A1. Detection of PCPE in murine tissue extracts by SDS-PAGE and western blotting. 100μg protein was loaded in each lane. Electrophoresis was carried out in reducing conditions with 10% acrylamide in the separating gel. Antibody detection (anti-36kDa PCPE; 1:500 dilution) was by enhanced chemiluminescence.

Appendix 2

Proposed Model for TRAMP-Collagen Interactions

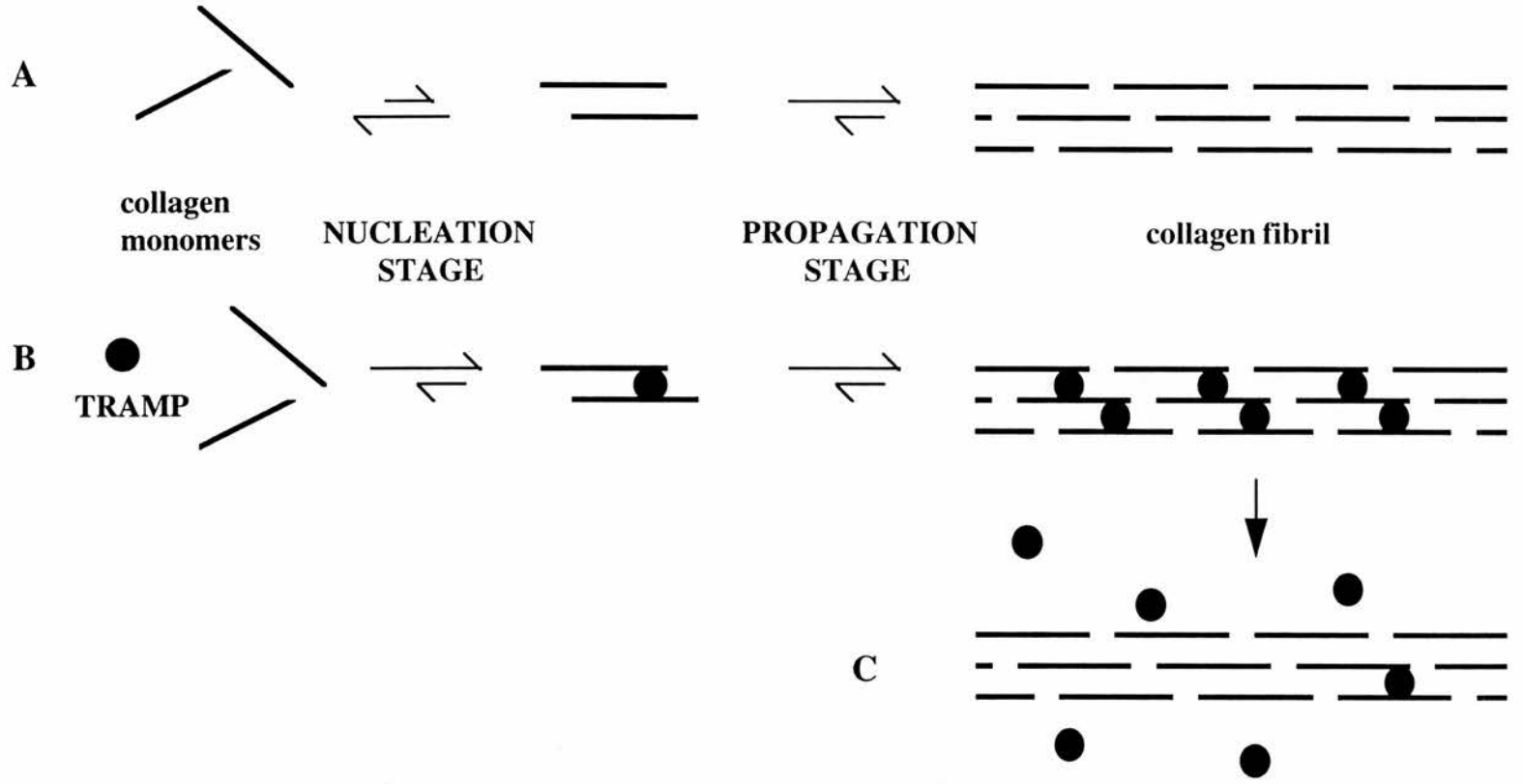


Figure A2: Proposed model to explain the higher affinity of TRAMP for collagen I monomers compared to fibrils. In the absence of TRAMP (A) the limiting step in the formation of collagen fibrils is the formation of the initial nucleus (in this case visualised as a dimer). When TRAMP is present (B) it would bind two neighbouring monomers thus stabilising the nucleus and shifting the equilibrium of the nucleation stage to the right. Although TRAMP would be incorporated into the mature fibril, its low binding affinity for fibrils would mean TRAMP would be easily displaced (C). If this was the case *in vivo* TRAMP would only be present at the growing ends of collagen fibrils and may thus also serve to direct fibroblasts to sites for collagen deposition.

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